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Original article

Synthesis, cytotoxicity and apoptosis of cyclotriphosphazene compounds as anti-cancer agents

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ABSTRACT

In the present study, a number of new dispirobino and dispiroansa spermine derivatives of cyclotriphosphazene (**8**–**10**, **13**) were synthesized and characterized by elemental analysis, mass spectrometry, ^{1}H and ^{31}P NMR spectroscopy. At first, *in vitro* cytotoxic activity of cyclotriphosphazene compounds (**1**–**14**) against HT-29 (human colon adenocarcinoma), Hep2 (Human epidermoid larynx carcinoma), and Vero (African green monkey kidney) cell lines was investigated. Our study showed that most of these compounds stimulate apoptosis and they have cytotoxic effects for HT-29 and Hep2 cells. Additionally, these compounds (**1**–**14**) were investigated for their antibacterial activity against gram-positive (*Staphylococcus aureus*), gram-negative (*Escherichia coli, Pseudomonas aeruginosa*) bacteria and for their antifungal activity against *Candida albicans*, and were shown to be inactive.

1. Introduction

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008 [1]. Treatment options for a cancer patient vary depending on the type of cancer, its location and the stage at which it is diagnosed. The administration of anti-cancer drugs is one of the effective cancer treatment options, and search for new anti-cancer agents is a great task as not only many cancer types exist but also the response of each individual to different anti-cancer drugs may vary considerably. Another challenge is to design chemicals that have minimum adverse effects on normal cells.

Many compounds with different structures have been tested by researchers in the search for anti-cancer drugs. Although a number of compounds exhibit therapeutical properties and are currently used for treatment, the search for new drugs with improved efficiency and minimum side effects still continues. Cyclotriphosphazene derivatives have attracted the attention of researchers to be used as potential anti-cancer agents. Interest in cyclotriphosphazenes as anti-cancer agents, previously mentioned by Labarre et al. [2], has been enhanced by the finding of aziridino- and polyamine-linked cyclotriphosphazenes which are active on a large series of tumour cells [3,4]. A number of studies have been devoted to describe the antitumour activity of cyclotriphosphazenes [5–8]. Cyclotriphosphazenes, having a variety of applications in science and technology, are an important class of inorganic ring system [9]. Chemical and physical properties of cyclotriphosphazenes change with the substituted side groups. So, it is possible to design materials with special properties such as anti-cancer agents [5–7], liquid crystals [10,11], electrical conductivity [12], hydraulic fluids and lubricants [13,14], flame retardant properties [15], elastomers [16], rechargeable batteries [17] and biomedical materials [18,19]. In addition, biological activities of cyclotriphosphazene derivatives against various bacterial and fungal species are known [20,21].

The native polyamines are essential growth factors for cells and polyamine derivatives have been shown to have cytotoxic activity against numerous human cancer cell lines (e.g., breast, prostate, etc) [22–24]. Polyamine-based small molecules have been developed for use as biochemical probes and as potential therapies for a variety of diseases [25]. The polyamines inhibit cell growth and

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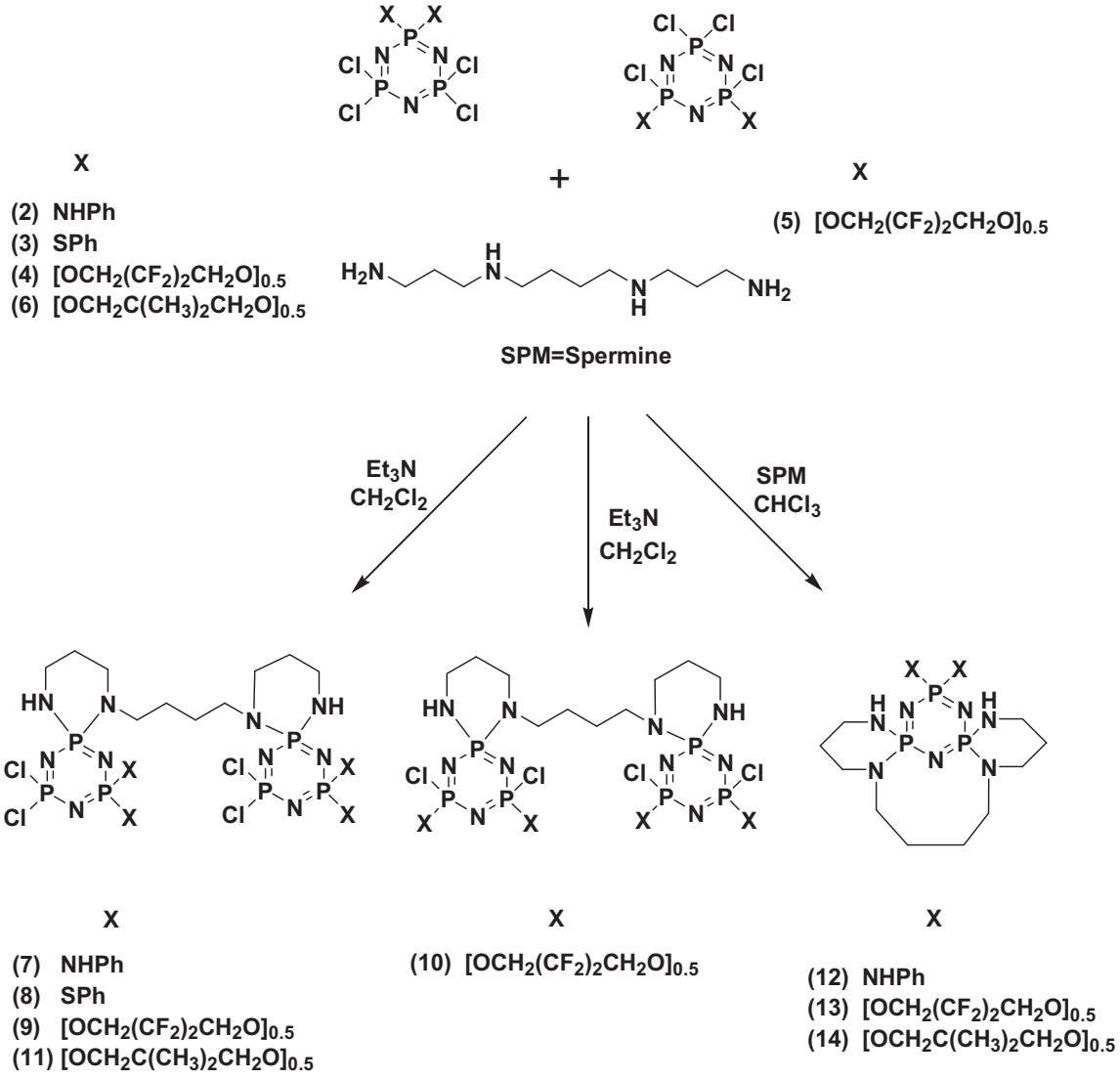
kill cancer cells both in tissue culture and experimental animal models [26–29]. One important property in the treatment of any cancer is to kill the tumour cells rather than just inhibiting their growth, although stable growth inhibition might be an acceptable alternative. In mammals, polyamines directly affect apoptosis in several cell types [25]. Polyamines are used in the protection of cells from external toxic conditions, such as oxidative stress, radiation, acidic pH, and other toxic agents [30]. In addition, antiparasitic and antibacterial effects of polyamine-linked analogues have been reported [31,32]. Spermine, found in all mammalian cells, can act as a regulator of DNA synthesis (replication, transcription, translation, and enzyme activities) and in the inhibition of cellular proliferation. Several studies have concluded that spermine-linked analogues affect gene expression at the transcriptional level and this effect is most probably due to the direct interaction of spermine with DNA and interacting protein factors [33–36].

In this study, hexachlorocyclotriphosphazene, $N_3P_3Cl_6$ (**1**), disubstituted derivatives of cyclotriphosphazene (**2**–**6**) and spermine derivatives of cyclotriphosphazene compounds (**7**–**14**) were synthesized and their cytotoxic properties against cell lines were investigated to assess their anti-cancer capabilities. Additionally, the antimicrobial effects of the compounds were assessed.

2. Results and discussion

2.1. Chemistry: synthesis and characterization of the compounds

Reaction of $N_3P_3Cl_6$ (**1**) with spermine aprotic solvents such as dichloromethane results in a series of dispirobino spermine derivatives of cyclotriphosphazene compounds, whereas, in protic solvents such as chloroform proceeds to yield the dispiroansa derivatives [37]. In this work, firstly disubstituted derivatives of cyclotriphosphazene (**2**–**6**) were synthesized from the reactions of **1** with the aniline, thiophenol, 2,2,3,3-tetrafluorobutane-1,4-diol, 2,2-dimethyl-1,3-propanediol, respectively [38–41]. And then, the reactions of gem-, spiro-, ansa-substituted cyclotriphosphazene derivatives $N_3P_3Cl_4R_2$ [R = NHPh, SPh, $(OCH_2(CF_2)_2CH_2O)_{0.5}$, $(OCH_2C(CH_3)_2CH_2O)_{0.5}$], (**2**–**6**) with spermine in dichloromethane results in dispirobino spermine derivatives of cyclotriphosphazene (**7**–**11**), namely spermine bridged compounds. But, if chloroform is used, the reaction results in dispiroansa derivatives (**12**–**14**), namely tetracyclic cyclotriphosphazene compounds (Scheme 1). The synthesis and characterization of compounds (**7**, **11**, **12**, **14**) were reported in our previous study [37], here we describe the biological activities of these compounds (**7**, **11**, **12**, **14**) and novel compounds (**8**–**10**, **13**).



Scheme 1. Dispirobino and dispiroansa spermine derivatives of cyclotriphosphazenes.

The exact synthesis of these compounds (**8**–**10**, **13**) is described in the Experimental section and graphical presentation is given in Scheme 1. Each of the new compounds (**8**–**10**, **13**) has been characterized by MS, elemental analysis, ^{1}H and ^{31}P NMR spectroscopy. The ^{1}H NMR results are provided as part of the analytical data in the synthesis section. Spermine bridged analogues with gem-substituted cyclotriphosphazene rings (e.g., compounds **8**, **9**) exhibit stereoisomerism [37] because the three phosphorus atoms of each cyclotriphosphazene ring have different substitution patterns and those that are part of the bridge, >P(N-spiro), are stereogenic, i.e., there are *R* and *S* forms. As seen from the Table 1, the proton decoupled ^{31}P NMR spectrum of compound **8** is observed as AMX and compound **9** is observed as ABX spin system due to the different environments for the three different phosphorus nuclei of the cyclotriphosphazene ring. The ^{31}P NMR spectra of compounds **10**, **13** are analysed as A_2X and AX_2 spin systems, respectively.

2.2. Biology

2.2.1. The cytotoxic effects of cyclotriphosphazene compounds

In this study, cyclotriphosphazene compounds were tested for their cytotoxic activity *in vitro* against human colon adenocarcinoma (HT-29), human epidermoid larynx (Hep2) cancer cell lines and as control African green monkey kidney (Vero) cell line. In order to test the cytotoxic effects, the cells were incubated with cyclotriphosphazene compounds at different concentrations (0.2–50 μg/mL) for 24 h at 37 °C. The cytotoxic effects of these compounds were determined by MTT assay (Table 2).

Probit analysis was used to evaluate the data and the calculated ID_{50} values (the dose of compound that inhibits cell proliferation by 50%) of the compounds against cell lines can be found from Table 2. Cytotoxicity of **1**, **3**–**6**, **8**, **9**, **11**, **12** compounds against HT-29 cells was detected after incubation for 24 h. Under the same conditions, cytotoxicity was detected on Hep2 cells after incubation with the compounds **3**–**6**, **10**, **13**, **14**. However, compounds **2**, **7**, **10**, **13**, **14** did not exhibit any cytotoxic effect on the HT-29 cells and the compounds **1**, **2**, **7**–**9**, **11**, **12** did not show any cytotoxic effect on the Hep2 cells. These results showed that cyclotriphosphazene compounds have selective and significant effect on the cell lines. For comparison, activity of compounds (**1**–**14**) against normal cells (Vero) was also examined. The anti-proliferative activity of some compounds (**1**–**7**, **9**–**11**) was much higher against the cancer cells than the normal cells. Although the compounds **8**, **12**–**14** had cytotoxic effects on HT-29 and Hep2 cells, they did not have any effect on the Vero cells.

Table 2
In vitro cytotoxic activity of the cell lines were examined by MTT assay after treating HT-29, Hep2 and Vero cells with varying concentrations of cyclophosphazenes compounds (0.2–50 μg/mL) for 24 h. The obtained data were evaluated using Probit analysis and described as ID_{50} values. ('Negative' in the table shows the compounds that have no toxic effect for the stated cell line.).

Compound	Cell Line ID_{50} [μM] ± SE		
	HT-29	Hep2	Vero
	ID_{50} ± SE	ID_{50} ± SE	ID_{50} ± SE
1	7.14 ± 0.05	Negative	3.16 ± 0.03
2	Negative	Negative	Negative
3	3.78 ± 0.03	5.43 ± 0.07	3.02 ± 0.03
4	4.31 ± 0.03	3.30 ± 0.05	3.55 ± 0.05
5	5.59 ± 0.05	2.70 ± 0.05	5.84 ± 0.05
6	4.09 ± 0.03	2.30 ± 0.03	2.96 ± 0.03
7	Negative	Negative	Negative
8	1.96 ± 0.01	Negative	3.35 ± 0.03
9	2.33 ± 0.02	Negative	1.31 ± 0.01
10	Negative	2.63 ± 0.03	1.61 ± 0.01
11	2.75 ± 0.02	Negative	2.65 ± 0.02
12	4.58 ± 0.04	Negative	6.55 ± 0.06
13	Negative	3.39 ± 0.04	5.41 ± 0.04
14	Negative	3.23 ± 0.04	5.88 ± 0.06

2.2.2. Induction of apoptotic cell death in cells by the compounds

Flow cytometry was used to investigate analogue-induced apoptosis and cell death. The compounds (**1**–**14**) with cytotoxic effects were investigated for the effects on the apoptosis of HT-29 and Hep2 cells. To clarify the mechanism of cyclotriphosphazene-induced cell death in cells, we determined both early and late apoptosis using annexin V-FITC and PI (Propidium iodide) labelling of live cells. Annexin V binds to phosphatidylserine which is exposed on the cell membrane and it is one of the earliest indicators of cellular apoptosis. PI is used as a DNA stain for both flow cytometry to evaluate cell viability or DNA content in cell cycle analysis and microscopy to visualise the nucleus and other DNA

Table 1
^{31}P NMR parameters of compounds **2**–**14**.[a]

Chemical Shifts/ppm					*R*	^{2}J(PP)/Hz			Ref.
Compound	P(N) Spiro	PCl(R)	PR_2	PCl_2		$^{2}J_{1,2}$	$^{2}J_{1,3}$	$^{2}J_{2,3}$	
2[b]	–	–	−1.1	23.0	NHPh	–	49.2	––	[38]
3[c]	–	–	48.8	20.3	SPh	–	5.4	–	[42]
4[b]	–	–	9.3	25.9	$[OCH_2C(CF_2)_2CH_2O]_{0.5}$	–	74.3	–	[40]
5[d]	–	24.1	–	25.6	$[OCH_2C(CF_2)_2CH_2O]_{0.5}$	–	48.0	–	[40]
6[b]	–	–	3.2	24.5	$[OCH_2C(CH_3)_2CH_2O]_{0.5}$	–	68.7	–	[41]
7[e,h]	14.9	–	2.9	24.1	NHPh	38.1	57.2	44.5	[37]
8[e,h]	13.2	–	46.5	22.6	SPh	6.2	9.2	36.3	This work
9[f,h]	15.4	–	15.7	26.7	$[OCH_2C(CF_2)_2CH_2O]_{0.5}$	57.1	63.1	–	This work
10[b]	15.7	28.1	–	–	$[OCH_2C(CF_2)_2CH_2O]_{0.5}$	–	51.6	–	This work
11[e,h]	15.9	–	9.4	25.1	$[OCH_2C(CH_3)_2CH_2O]_{0.5}$	37.0	69.9	64.4	[37]
12[b]	17.0	–	9.4	–	NHPh	–	–	50.8	[37]
13[c]	17.8	–	24.4	–	$[OCH_2C(CF_2)_2CH_2O]_{0.5}$	–	–	71.6	This work
14[g]	18.0	–	17.7	–	$[OCH_2C(CH_3)_2CH_2O]_{0.5}$	–	–	54.7	[37]

[a] 202.38 MHz ^{31}P NMR measurements in $CDCl_3$ solutions at 298 K. Chemical shifts referenced to external H_3PO_4.
[b] ^{31}P NMR spectrum analysed as an A_2X spin system.
[c] ^{31}P NMR spectrum analysed as an AX_2 spin system.
[d] ^{31}P NMR spectrum analysed as an AB_2 spin system.
[e] ^{31}P NMR spectrum analysed as an AMX spin system.
[f] ^{31}P NMR spectrum analysed as an ABX spin system.
[g] ^{31}P NMR spectrum analysed as an A_2B spin system.
[h] Diastereoisomer.

containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells.

In the present study, the apoptotic cell rates were determined for the HT-29 cells that were stimulated for 24 h by the compounds (**1**, **4**–**6**, **9**, **11**, **12**) with cytotoxic effects at different concentrations. The graphical values of the results were given in Fig. 1. From the results, it was found that these compounds stimulate apoptosis for the HT-29 cells compared to positive (etoposide) and negative (unstimulated cells by compounds) controls, for example compounds **1**, **9** and **12** induced apoptosis in 80% of the cells. As seen with the control compound (etoposide), most of the cytotoxic anti-cancer drugs in current use induce apoptosis in susceptible cells [47]; Compounds **4** and **5**, however, are of special interest as these compounds seem to drive HT-29 cells directly from early apoptosis into necrosis. The fact that the cell populations are so cleanly sequestered into the upper left (necrosis) and lower right quadrants (early apoptotic cells) is interesting. Either these compounds give a divergent result where some cells are killed directly (necrosis) and some are induced to undergo apoptosis, or they speed up the apoptosis process so that we start observing necrosis before the test ends and perhaps if time allowed others would have followed soon.

The apoptotic cell rates were determined in the Hep2 cells that were stimulated for 24 h by the compounds (**4**–**6**, **13**, **14**) with cytotoxic effects at different concentrations and the results shown in Fig. 2. From these compounds **4**, **6** and **14** were determined to stimulate apoptosis compared to positive (cisplatin) and negative controls. The results obtained from compound **6** attract extra attention as it stimulates apoptosis for 92% of the Hep2 cells.

The majority of drugs used for the treatment of cancer today are cytotoxic drugs that induce apoptosis in susceptible cells. Over the last fifty years thousands of chemical compounds have been tested for anti-cancer activity, but only a few of these are being used for cancer treatment and are in wide use today. Therefore, studies in this area continue to grow in importance. Some of the compounds synthesized in the study attract particular attention as anti-cancer agents (**1**, **4**, **5**, **9**, **12** for HT-29 cells and **4**, **6**, **14** for Hep2 cells) and further studies are required to explore their potential in this area.

2.2.3. Antimicrobial activities

Infectious diseases caused by *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* microorganisms represent a serious threat to the health of hospitalized

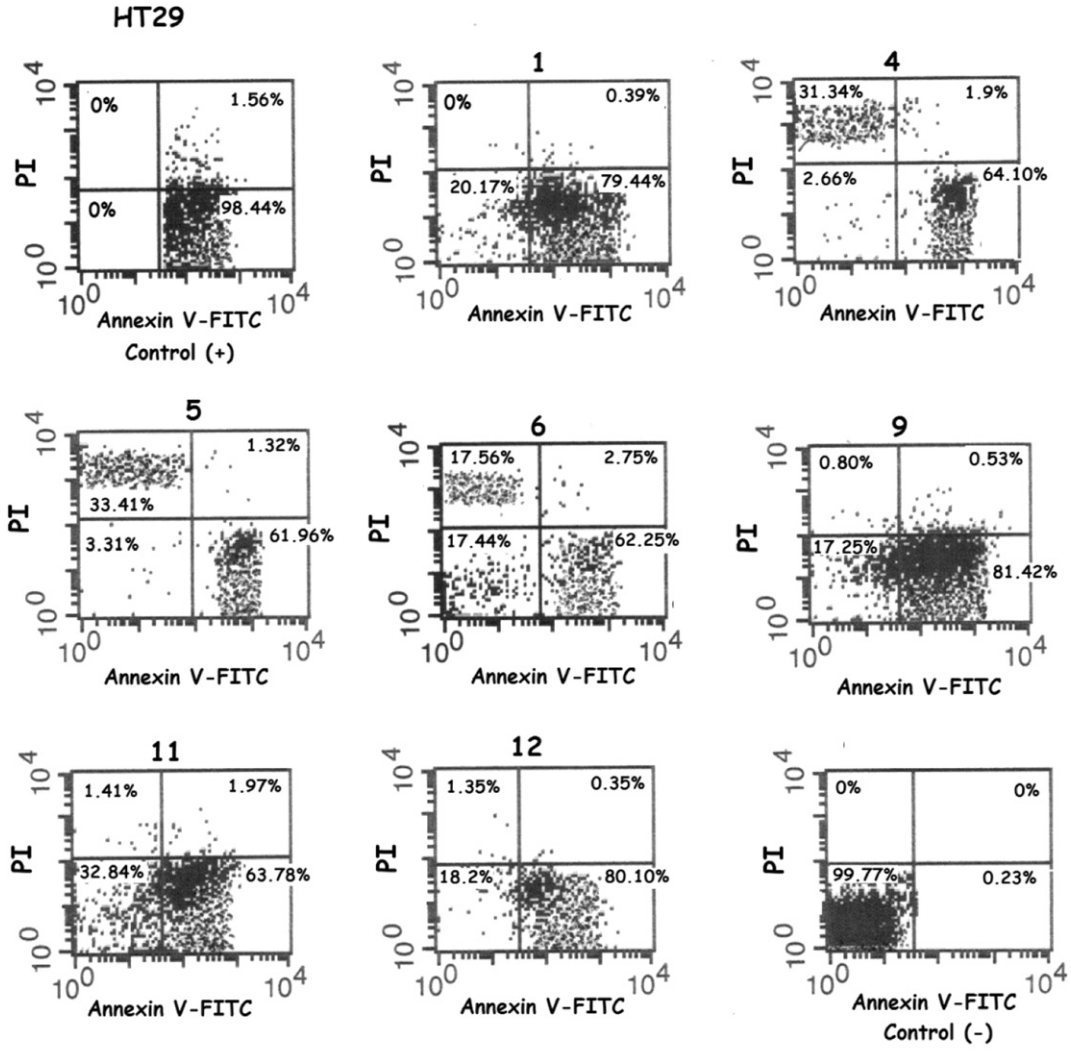


Fig. 1. Effect of cyclotriphosphazene compounds on apoptosis of HT-29 cells. Apoptotic cells were analyzed by flow cytometry, after being stained with Annexin V-FITC together with PI. The percentage of cells positive for PI and/or Annexin V-FITC are reported inside the quadrants. Lower left quadrant, viable cells (annexin V−/PI−); lower right quadrant, early apoptotic cells (annexin V+/PI−); upper right quadrant, late apoptotic cells (annexin V+/PI+); upper left quadrant, necrotic cells (annexin V−/PI+). The percentage of cells positive for PI and/or Annexin V-FITC was reported inside the quadrants.

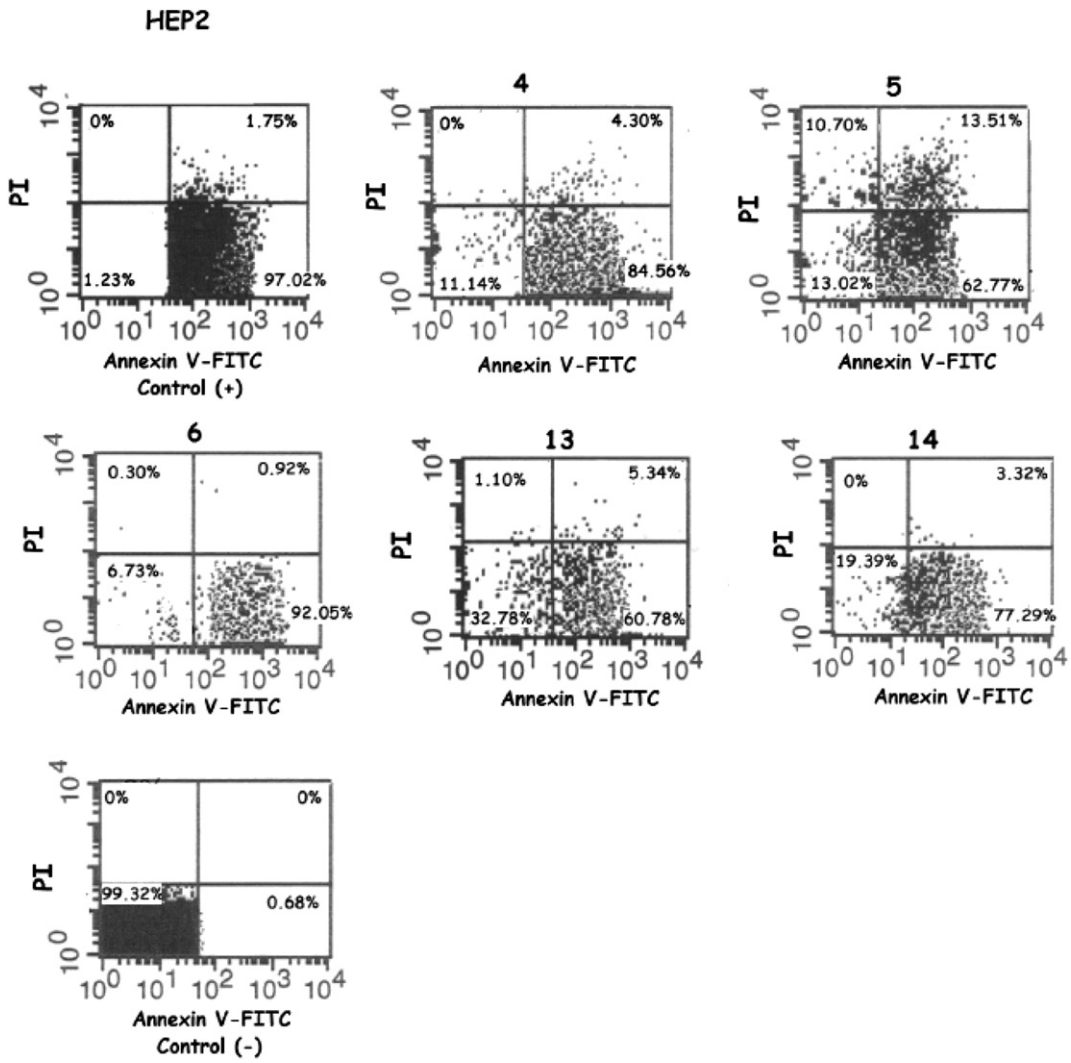


Fig. 2. Effect of cyclotriphosphazene compounds on apoptosis of Hep 2 cells. Apoptotic cells were analyzed by flow cytometry, after being stained with Annexin V-FITC together with PI. The percentage of cells positive for PI and/or Annexin V-FITC are reported inside the quadrants.

patients. The discovery of new antimicrobial agents against these microorganisms has great importance, therefore, in the study the antimicrobial effects of the newly synthesized cyclotriphosphazene compounds were investigated as no previous reference has been found related with the biologic activity of these compounds.

All these compounds (**1**–**14**) were investigated for their *in vitro* antibacterial activities against *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 35218) and *S. aureus* (ATCC 25923), and for antifungal activities against *C. albicans* (ATCC 10231). The minimum inhibitory concentration (MIC) values of these compounds were determined by the broth microdilution method (range, 2–1024 μg/mL). All the compounds investigated gave an ID_{50} above 250 μg/mL, the high MIC values of these compounds indicated that the synthesized cyclotriphosphazene compounds do not have any significant antimicrobial activity against standard strains of gram-positive *S. aureus*, gram-negative *E. coli*, *P. aeruginosa* and yeast like fungi *C. albicans.*

3. Conclusions

Cancer is a leading cause of death worldwide and hence studies to find anti-cancer agents for its treatment continue to grow in importance. In the present study, as potential anti-cancer agents, disubstituted derivatives (**2**–**6**) and spermine derivatives of cyclotriphosphazene compounds (**7**–**14**) were synthesized and characterized by elemental analysis, mass spectrometry, ^{1}H and ^{31}P NMR spectroscopy. Later the cytotoxic and apoptotic effects of the cyclotriphosphazene compounds on cancer cell lines were determined. It was detected that the Compounds **1**, **4**, **5**, **9**, **12** and **4**, **6**, **14** have selective and significant cytotoxic effects on HT-29 and Hep2 cells, respectively. Therefore, these compounds may be considered as the agents with highest potential anti-cancer activity and appear to be good candidates for more advanced screening. As the findings of this work revealed the anti-cancer properties of specific cyclotriphosphazene derivatives, investigation of these materials will continue in our laboratories.

4. Experimental

4.1. Chemistry

Hexachlorocyclotriphosphazene (Otsuka Chemical Co., Ltd) was purified by fractional crystallization from hexane. Tetrahydrofuran (≥99.0%), n-hexane (≥96.0%), methanol (≥99.0%), sodium hydride

(60% dispersion in mineral oil), 2,2-dimethyl-1,3-propanediol (≥98.0%), dichloromethane (≥99.0%), aniline (≥99.0%), chloroform (≥99.0–99.4%), triethylamine (≥99.0%), thiophenol (≥99.0%) were obtained from Merck. Spermine (≥99.0%) was obtained from Fluka. 2,2,3,3-tetrafluoro-1,4-butanediol (≥98.0%) was obtained from Aldrich. All solvents were purified by conventional methods. THF was distilled over a sodium–potassium alloy under an atmosphere of dry argon. $CDCl_3$ for NMR spectroscopy was obtained from Merck. Elemental analyses were obtained using a Thermo Finnigan Flash 1112 Instrument. Mass spectra were recorded on a Bruker MicrOTOF LC-MS spectrometer using the electro spray ionization (ESI) method; ^{35}Cl values were used for calculated masses. Analytical Thin Layer Chromatography (TLC) was performed on Merck silica gel plates (Merck, Kieselgel 60, 0.25 mm thickness) with F_{254} indicator. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 230–400 mesh; for 3 g crude mixture, 100 g silica gel was used in a column of 3 cm in diameter and 60 cm in length). ^1H and ^{31}P NMR spectra were recorded in $CDCl_3$ solutions on a Varian INOVA 500 MHz spectrometer using TMS as an internal reference for ^1H NMR and 85% H_3PO_4 as an external reference for ^{31}P NMR.

Compounds (**2**–**7**, **11**, **12**, **14**) were prepared as described in the literature [38–41].

4.1.1. Synthesis of compound *8*

Compound **3** [39] (2.0 g, 4.0 mmol) and triethylamine (2.3 mL, 17.2 mmol) were dissolved in 100 mL of dichloromethane in a 250 mL three-necked round-bottomed flask. The reaction mixture was cooled in an ice-bath and spermine (0.8 g, 4.0 mmol) in 10 mL of dichloromethane was added to the stirred solution under an argon atmosphere. The reaction was stirred for four days at room temperature and followed by TLC on silica gel plates with *n*-hexane-THF (3:1) as mobile phase. The reaction mixture was filtered to remove the triethylamine hydrochloride, the dichloromethane was removed and the resulting colourless oil subjected to column chromatography, using *n*-hexane-THF (3:1) as mobile phase. Compound **8** was isolated as oil (1.5 g, 72%). *Anal* Calc. $C_{34}H_{42}Cl_4N_{10}P_6S_4$ requires: C, 39.02; H, 4.04; N, 13.38%, M, 1046, Found: C, 39.00; H, 4.01; N, 13.31%, $[M-H+Na]^+$,1069. ^1H NMR, $CDCl_3$, 298 K: 7.9–7.3 m, 20H, Ar–C<u>H</u>; 4.2m, 4H, NHC<u>H</u>$_2$; 3.7–2.8 m, 8H, NC<u>H</u>$_2$; 2.7 broad, 2H, N<u>H</u>; 1.7–2.1 m, 8H, CC<u>H</u>$_2$ (spiro and bridge).

4.1.2. Synthesis of compound *9*

Compound **4** [40] (2.0 g, 4.6 mmol) and triethylamine (2.6 mL, 18.4 mmol) were dissolved in 100 mL of dichloromethane in a 250 mL three-necked round-bottomed flask. The reaction mixture was cooled in an ice-bath and spermine (0.9 g, 4.6 mmol) in 10 mL of dichloromethane was added to the stirred solution under argon atmosphere. The reaction was stirred for 23 h at room temperature and followed by TLC on silica gel plates *n*-hexane-THF (3:2) as mobile phase. The reaction mixture was filtered to remove the triethylamine hydrochloride formed, the dichloromethane was removed and the resulting colourless oil product subjected to column chromatography using *n*-hexane-THF (3:2) as mobile phase. Compound **9** was isolated as a white powder (1.9 g, 45%, mp >250 °C). *Anal* Calc. $C_{18}H_{30}Cl_4F_8N_{10}O_4P_6$ requires: C, 23.24; H, 3.25; N, 15.06%, M, 930, Found: C, 23.20; H, 3.21; N, 15.00%, $[M+H]^+$, 931. ^1H NMR, $CDCl_3$, 298 K: 4.3–4.2 m, 8H, OC<u>H</u>$_2$, 4.2–4.1 4H, NHC<u>H</u>$_2$; 3.9–2.8 m, 8H, NC<u>H</u>$_2$; 2.80 broad, 2H, N<u>H</u>; 2.0–1.6 m, 8H, CC<u>H</u>$_2$ (spiro and bridge).

4.1.3. Synthesis of compound *10*

Compound **5** [40] (0.4 g, 0.9 mmol) and triethylamine (0.5 mL, 3.7 mmol) were dissolved in 40 mL of dichloromethane in a 100 mL three-necked round-bottomed flask. The reaction mixture was cooled in an ice-bath and spermine (0.2 g, 0.9 mmol) in 10 mL of dichloromethane was added to the stirred solution under argon atmosphere. The reaction was stirred for 23 h at room temperature and followed by TLC on silica gel plates using *n*-hexane-THF (2:1) as the mobile phase. The reaction mixture was filtered to remove the triethylamine hydrochloride formed, the dichloromethane was removed and the resulting colourless oil product subjected to column chromatography using *n*-hexane-THF (2:1) as mobile phase. Compound **10** was isolated as white powder (0.3 g, 35%, mp 214–216 °C). *Anal* Calc. $C_{18}H_{30}Cl_4F_8N_{10}O_4P_6$ requires: C, 23.24; H, 3.25; N, 15.06%, M, 930, Found: C, 23.21; H, 3.22; N, 15.00%, $[M+H]^+$, 931. ^1H NMR, $CDCl_3$, 298 K: 4.1–4.0 m, 8H, OC<u>H</u>$_2$, 3.8–3.5 4H, NHC<u>H</u>$_2$; 3.9–2.7 m, 8H, NC<u>H</u>$_2$; 2.8 broad, 2H, N<u>H</u>; 2.3–1.6 m, 8H, CC<u>H</u>$_2$ (spiro and bridge).

4.1.4. Synthesis of compound *13*

Compound **4** [40] (1.0 g, 2.3 mmol) was dissolved in 50 mL of $CHCl_3$ in a 250 mL three-necked round-bottomed flask. The reaction mixture was cooled in an ice-bath and spermine (0.9 g, 4.6 mmol) in 20 mL of chloroform was added to the stirred solution under an argon atmosphere. The reaction was stirred for 23 h at room temperature and followed by TLC on silica gel plates using THF-dichloromethane (2:1) as the mobile phase. The reaction mixture was filtered to remove the spermine tetrahydrochloride formed, the chloroform was removed under reduced pressure and the resulting colourless oil was subjected to column chromatography using THF-dichloromethane (2:1) as mobile phase. Compound **13** was isolated as white powder (0.6 g, 53%, mp 250–251 °C). Anal Calc. $C_{14}H_{26}F_4N_7O_2P_3$ requires: C, 34.09; H, 5.31; N, 19.87%, M, 493, Found: C, 34.05; H, 5.28; N, 19.81%, $[M+H]^+$, 494. ^1H NMR, $CDCl_3$, 298 K: 4.5–4.3 m, 4H, OC<u>H</u>$_2$, 3.9 4H, NHC<u>H</u>$_2$; 2.9–2.8 m, 8H, NC<u>H</u>$_2$; 2.81 broad, 2H, N<u>H</u>; 1.8–1.5 m, 8H, CC<u>H</u>$_2$ (spiro and ansa).

4.2. Biological assays

4.2.1. Cytotoxicity of the compounds

Cell Lines. The following *in vitro* human cancer cell lines were used: HT-29 (human colon adenocarcinoma), Hep2 (Human epidermoid larynx carcinoma) and Vero (African green monkey kidney). The cell lines (HT-29/An1-9704220, Hep2/An1-92041501, Vero/An10-97121501) were purchased from the Institute of Foot and Mouth Diseases, Virology Laboratory, Ankara, Turkey.

Cell Culture. HT-29 and Vero cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (Sigma, Steinheim, Germany) containing 2 mM Na_2CO_3 supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma, Steinheim, Germany). Hep2 cells were cultured in EMEM (Sigma, Steinheim, Germany) and 10% (v/v) of foetal bovine serum. The cell culture media was supplemented with penicillin/streptomycin at 100 Units/mL (Sigma, Steinheim, Germany) as adherent monolayers. Cell lines were incubated at 37 °C under 5% CO_2 and 95% air in a humidified atmosphere. Stock solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany) and further dilutions were made with fresh culture medium. The concentration of DMSO in the final culture medium was 1%, which had no effect on the cell viability.

MTT assay. The cytotoxic response to the cell lines and the growth-inhibitory effects of compounds were determined by MTT assay using a colorimetric substrate tetrazolium salt, 3-[4,5-dimethyl-thiazollyl-2-yl]-2,5-diphenyltetrazolium bromide (AppliChem, Darmstadt, Germany) [43]. Hep2 (2×10^4 cells/mL), Vero (2×10^4Darmstadtcells/mL) and HT-29 (3×10^4Darmstadtcells/mL) cells were incubated with the compounds (0.2–50 μg/mL) in 96-well plates (TPP, Switzerland) for 24 h. After incubation, culture

medium was removed and 100 μL of MTT reagent (1 mg/mL in serum free medium) was added to each well. The plates were incubated for 2 h at 37 °C. MTT containing medium was removed and each well was washed gently with 100 μL of PBS. The blue formazan product was dissolved by addition of 100 μL of 100% DMSO per well. The plates were swirled gently for 10 min to dissolve the precipitate, and quantified by measuring the optical density (OD) of the plates at a wavelength of 540 nm on a micro plate reader (Bio-Tek ELX808 IU). The mean OD values for the negative control (DMSO treated cells) were standardized as 100% OD (i.e. no growth inhibition) and results were displayed as OD (% of control) concentrations of the compounds. ID_{50} value is the concentration of compounds required to inhibit growth by 50%. Each concentration was repeated in three wells and control cell viability was considered as 100%. The same experimental conditions were provided for all compounds and MTT analysis was repeated three times for each cell line.

Determination of apoptosis. Apoptosis or necrosis was discriminated with the annexin V-FITC/propidium iodide test [44]. Cells were seeded at $2 - 3 \times 10^6$/well in 10% FBS–DMEM into 75 cm^2 tissue culture flasks (Greiner, Bio-one) and treated with compounds for 24 h. Phosphatidylserine externalisation was determined by annexin V staining. Culture flasks were washed with PBS 3 times. Cells were pelleted and re-suspended in 1 mL annexin binding buffer (10 mM HEPES [pH: 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$) (Sigma, Steinheim, Germany). The cells were stained with 100 ng/mL annexin V-FITC-conjugated (fluorescein isothiocyanate) (BD, Pharmingen) and 0.1 μg/mL of propidium iodide (PI) (Calbiochem, USA). Cells were incubated for 15 min at room temperature in the dark and cells were analyzed with a flow cytometer (FACSCalibur, BD Biosciences). Etoposide (12.5–25–50 μg/mL) (Calbiochem, Darmstadt, Germany) and cisplatin (200 μM) (Sigma, Steinheim, Germany) were used as internal positive controls. The spectral overlap was electronically compensated for with single-colour control cells stained with annexin V-FITC or PI. Analysis was performed with the system software (CellQuest; BD Biosciences). Lower left quadrant, viable cells (annexin V−/PI−); lower right quadrant, early apoptotic cells (annexin V+/PI−); upper right quadrant, late apoptotic cells (annexin V+/PI+); upper left quadrant, necrotic cells (annexin V−/PI+). The percentage of cells positive for PI and/or Annexin V-FITC was reported inside the quadrants. The experiment was repeated three times in duplicate, with similar results.

4.2.2. Determination of antibacterial activity

Antibacterial activities of the compounds (**1**–**14**) were determined by broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) [45]. In this study, *S. aureus* (ATCC 25923), *E. coli* (ATCC 35218), *P. aeruginosa* (ATCC 27853) standard strains were tested. Compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany) and sterilized by membrane filter. Compounds were tested at 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 μg/mL concentrations. Standard strains were incubated on blood agar plates at 37 °C for 18–24 h. After incubation, bacterial suspensions were adjusted to a turbidity of 0.5 McFarland and diluted 1:100 in Mueller Hinton Broth (MHB) (Oxoid, Hampshire, England). 100 μL of inoculums were inoculated into each well. The plates were incubated at 35 °C for 18–24 h. All tests were performed twice. MIC's were defined as the lowest concentrations of derivatives that inhibited growth of bacteria.

4.2.3. Determination of antifungal activity

Antifungal activities of the compounds (**1**–**14**) were determined by broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) [46]. In this study, yeast-like fungi *C. albicans* (ATCC 10231) standard strain was tested. Compounds were dissolved in dimethyl sulfoxide and sterilized by membrane filter. Compounds were tested at 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 μg/mL concentrations. Standard strains were incubated on Sabouraud dextrose agar (Merck, Darmstadt, Germany) plates at 37 °C for 48 h. After incubation, the yeast suspension was adjusted to a turbidity of 0.5 McFarland. Yeast suspension was diluted serially by 1:50 and then 1:20 in RPMI 1640 media (Sigma, Steinheim, Germany). 50 μL of inoculum were inoculated into each well. The plates were incubated at 35 °C for 48 h. All tests were performed twice. MIC's were defined as the lowest concentrations of compounds at which 50% of the fungi were inhibited compared to the positive control.

4.3. Statistics

Statistical analyses were performed with SPSS 18.0 for Windows. Probit analysis was used to detect the value of ID_{50}. The results were shown as $ID_{50} \pm$ standard error of mean (SE) for three cell lines.

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