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Ruthenium metallodendrimers with anticancer potential in an acute promyelocytic leukemia cell line (HL60)

Sylwia Michlewska [a], Maksim Ionov [b,*], Dzmitry Shcharbin [c], Marta Maroto-Díaz [d,e], Rafael Gomez Ramirez [d,e], Francisco Javier de la Mata [d,e], Maria Bryszewska [b]

[a] Laboratory of Microscopic Imaging and Specialized Biological Techniques, Faculty of Biology and Environmental Protection, University of Lodz, Banacha12/16, 90-237 Lodz, Poland
[b] Department of General Biophysics, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, 90-236 Lodz, Poland
[c] Institute of Biophysics and Cell Engineering of NASB, Akademicheskaja 27, Minsk 220072, Belarus
[d] Departamento Química Orgánica y Química Inorganica, Universidad de Alcala de Henares, CIBER-BBN, Alcala de Henares, Spain
[e] Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain

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ABSTRACT

Ruthenium belongs to the family of metals widely used in anticancer therapy (platinum, arsenic, antimony, bismuth, gold, vanadium, iron, rhodium, titanium, gallium). This paper deals with a new kind of ruthenium terminated carbosilane dendrimers (CRD), consisting of carbosilane dendrons functionalized with N-, NH_2-donor monodentate and N,N-chelating ruthenium complexes. The biophysical characterization, hemolytic activity and the cytotoxicity towards cancer (HL-60) and normal (B-14) cell lines of CRDs have been determined. The data indicate that: (1) generation 0 metallodendrimers are the most effective drugs, being non-toxic for normal cells but induced significant cytotoxicity (>90%) in cancer cells; (2) an increase of generation leads both increased cytotoxicity of CRDs and the levelling of the difference in their action on normal and cancer cells; (3) coordination modes of ruthenium in a dendrimer scaffold did not correlate with their cytotoxicity towards normal and cancer cells. Results suggest that ruthenium dendrimers can be considered as alternative anticancer therapeutic agent.

1. Introduction

In the development of better therapies for cancer, there are many effective medicines, but most are also toxic to healthy tissues. Platinum anticancer drugs are well known [1–3], but compounds based on this metal are highly toxic to many organisms [1,4]. Therefore, researchers are now focusing on drugs based on anticancer compounds containing metals such as silver, gold or ruthenium [1,2,5–8]; drugs based on these metals might be less toxic than platinum drugs [8]. In this sense, one of the most important advantages of ruthenium is that, in the living cells, it behaves like iron. This metal can have II, III or IV oxidation states under physiological conditions where a third oxidation stage is non-toxic to animal cells [7]. As iron, ruthenium can be bound to plasma proteins, such as albumin or transferrin [9,10]. Cancer cells possess more transferrin receptors than normal cells. Ruthenium can therefore be transported by proteins to tumour cells and internalized by endocytosis

* Corresponding author at: Department of General Biophysics, University of Lodz, Pomorska St. 141/143, Lodz 90-236, Poland.
E-mail address: maksion@biol.uni.lodz.pl (M. Ionov).
URL: http://www.biol.uni.lodz.pl (M. Ionov).

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instead of iron [11]. Inside the cancer cells, where pH is low, glutathione concentration can be high and ruthenium oxidation state can be reduced to II, which is highly cytotoxic [7]. In this state some ruthenium complexes can be bound to the DNA molecules, inhibiting their replication. Moreover, ruthenium can be mutagenic and reduce the RNA synthesis [3]. Finally, ruthenium can direct cells towards apoptosis [7–11].

The biological target for some ruthenium complexes has focused on 2 enzymes –thioredoxin reductase and cathepsin B, which both contain cysteine in their active site [12]. Additional benefits associated with the use of ruthenium compounds are that they do not seem to be toxic to normal cells [7].

The main problem with the delivery of ruthenium drugs is their poor solubility in water, which limits their transport and hence their use in anticancer therapy. Several carriers of anticancer drugs might help deliver them to cancer cells [13].

Nanotechnology is now an important field in oncology, interest being in particular focused mainly on the use of nanomaterials of controlled size, shape and surface functionality, bringing enhancement in specificity, activity and efficiency of drugs [14]. Dendrimers are a prime example because of their special their properties.

Dendrimers were first synthesized in the 1970s by the teams of Tomalia, Newcome and Vögtleteams. These synthetic branched polymers are currently used as drug carriers, increasing the bioavailability and solubility of drugs. Dendrimers are synthesized in a generation controlled manner [15,16], the nanoparticles being built with a centrally core from which polymer branches extend [5,6]. The exterior contains end groups that determine the behaviour of the dendrimer in solution [15–18]. One can also modify these surface groups. The polymers have a well-defined structure, a spherical shape and are monodisperse [4,15,19]. Moreover, their molecular weights can be accurately determined [20–22]. A significant part of dendrimers have low immunogenic activity and are mostly non-toxic [15,16]. The number of unique features makes them attractive for use in several fields of science, especially as drug delivery agents in medicine [4]. In particular, carbosilane dendrimers have features such as chemical and thermal stability, an inert framework, biocompatibility and a hydrophobic nature that might enhance interaction with biological membranes.

We have now attempted to characterize the anticancer properties of newly synthesized ruthenium-terminated carbosilane dendrimers. A combination of ruthenium anti-tumour activity with unique properties of dendrimers make these new compounds worthy of further investigation.

2. Results and discussion

2.1. Characterization of ruthenium-terminated carbosilane dendrimers

TEM showed whether morphological structure depends on dendrimer generation and number of surface groups in a dry state (Fig. 1), which shows that, in a dry state, they can form aggregates of different shapes and sizes. Electrostatic interactions occur between individual nanoparticles, therefore they can form aggregates [6]. Characteristic branched structures were seen for all the analysed dendrimers. The shapes and sizes of these aggregates depended on the nature of the dendrimer. The most homogenous formulations were seen for compound **CRD13** and **CDR27**dendrimers (Fig 1). The size of aggregates varied from 50 to 800 nm. Some single (non-aggregated) nanoparticles of 4–9 nm were also seen in the images, probably of single dendrimers. Because samples for TEM require their drying, this can influence the exact structure and distribution, reducing the accuracy of the information on CRD morphology.

Measurements of zeta potential of dendrimers in fluid solutions give information about their surface charges [23]. Values were determined for CRD dendrimers at 20 μmol/L in 10 mmol/L Na-phosphate buffered solution, pH 7.4. For the compounds investigated, charge values were positive at from 11.7 ± 2.3 to 57.6 ± 8.3 mV. Due to the negative charge of the plasma membrane, positively charged nanoparticles should easily interact with cells [17,24].

Dendrimers of generation 1 (**CRD14, CRD32**) and generation 2 (**CRD27**) had the highest zeta potentials (Table 1). These compounds appear more monodispersed and their size distribution varied from 211 ± 27.8 to 269 ± 36.1 nm (Table 1). The biggest sizes were detected for **CRD5** and **CRD13** of generation 0 at 1543 ± 154 and 456 ± 139 nm, respectively.

Size distribution was measured by DLS in solution, whereas TEM studies had been carried out on dry samples; they therefore give different estimates of dendrimer size [25]. Despite these differences, the methods corroborate that dendrimers can self-aggregate.

2.2. Hemotoxicity

Red blood cell lysis is a simple method of analysing membrane disruption [26]. Hemolysis assay was used to test the effect of dendrimers on the cell membrane. If the integrity of erythrocytes interacting with ligands is affected, there will be a release of their proteins, notably hemoglobin. Our dendrimers caused hemolysis after 24 h incubation (Fig. 2). The effect depended mostly on dendrimer generation and concentration. Dendrimers of generation 0 were least effective, with 31–38% haemolysis at 100 μmol/L. This can be explained by the small number of active surface groups of generation 0 CRDs. In contrast, hemolysis due to dendrimers of generations 1 and 2 was higher, with massive effectat25-50 μmol/L. The molar dependence of hemolysis could be due to the increased surface charge of the dendrimers, especially in the case of **CRD32** generation 1 (4 surface groups) and **CRD27** generation 2 (8 surface groups) (Fig. 3).

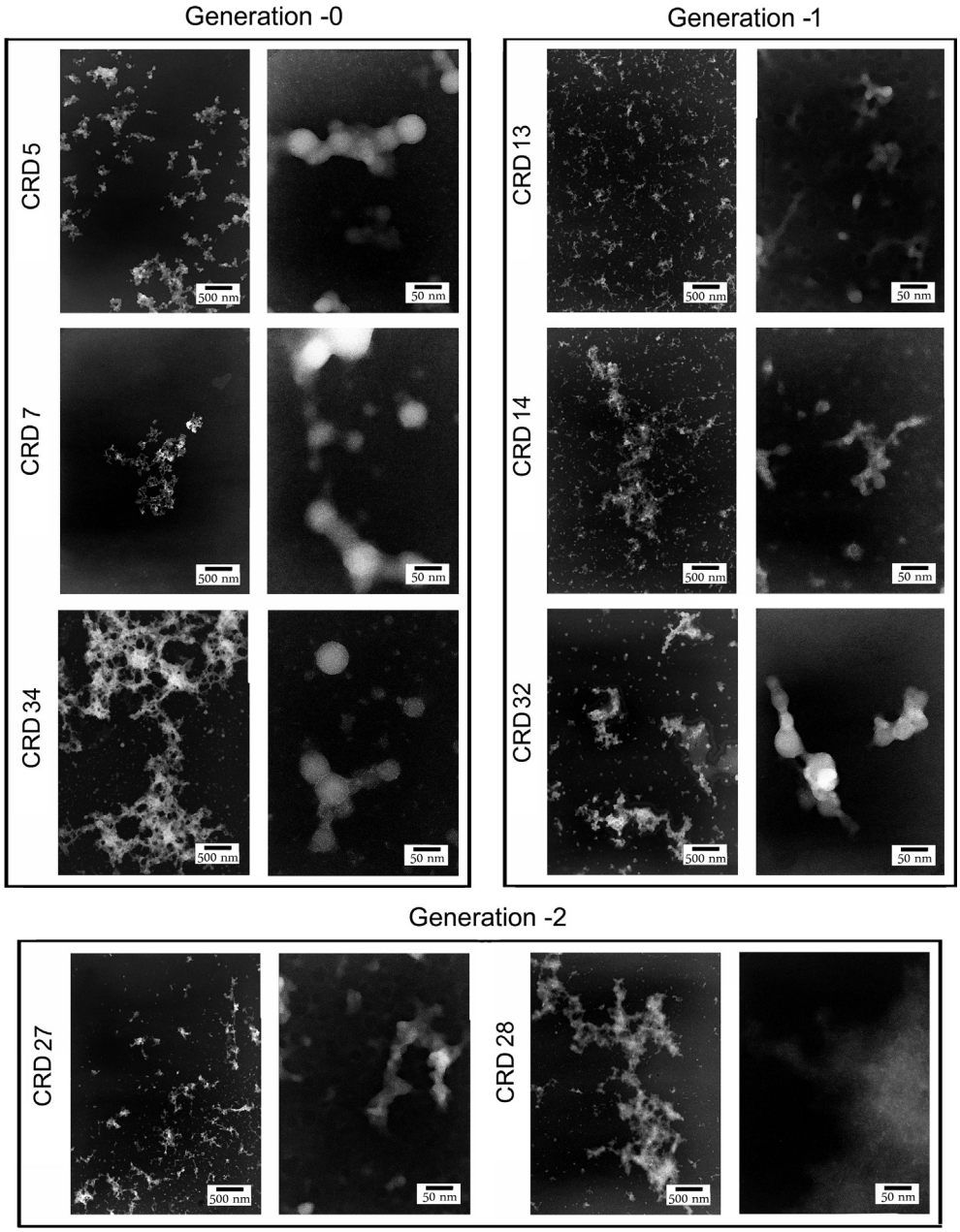


Fig. 1. Ultrastructure of ruthenium-terminated carbosilane dendrimers of generation 0 (**CRD7, CRD5, CRD34,** left panel), generation 1 (**CRD13, CRD14, CRD32**, right panel) and generation 2 (**CRD27, CRD28** bottom panel). Dendrimers were dissolved in Na-phosphate buffer 10 mmol/L, pH 7.4. Magnifications of 15,000 and 150,000×; bars = 50 nm and 500 nm [To to obtain greater contrast, the color of the micro images has been inverted].

Hemolytic activity of carbosilane dendrimers depends on their concentration [27]. A similar effect was seen with PAMAM dendrimers [28] and phosphorus dendrimers [29]. In contrast, hemolysis was not seen after 24 h incubation of red blood cells with titanium-containing mesoporous dendrimers [30]. An important factor that makes dendrimers toxic is therefore their surface charge [29].

Cationic dendrimers due to interaction with negatively charged membranes are more toxic than neutral or anionic dendrimers [15,31,32]. Numerous reports indicated that effect of polycationicpolymers: poly-L-lysine (PLL), polyethylenimine (PEI), diethylaminoethyl-dextran (DEAE-DEX), poly-amidoamine (PAMAM) on cell membrane permeabilization is directly proportional to number of positive functional groups [28,32–34]. In addition, the dendrimers can influence the hydrophobic part of the membranes, resulting the change of their fluidity [24]. Presented results demonstrated that ruthenium contained dendrimers can easily interact with biological membranes. From one hand higher generations of dendrimers with rising number of positively charged surface groups can increase the dendrimers toxicity. From other hand the bigger number of

Table 1
Zeta potential and zeta size of ruthenium-terminated carbosilane dendrimers of generations 0, 1 and 2.

Dendrimer	Z-potential, [mV]	SD[a]	Z-size, [nm]	SD[a]
CRD5	11.7	±2.3	543.4	±154.3
CRD7	12.4	±3.3	387.3	±126.1
CRD34	6.91	±3.1	320.7	±167.5
CRD13	21.5	±6.2	455.7	±139.0
CRD14	54.3	±6.7	269.9	±36.10
CRD32	12.7	±5.6	221.4	±193.2
CRD27	57.6	±8.3	211.5	±27.80
CRD28	44.3	±1.7	256.0	±96.10

[a] Means ± SD.

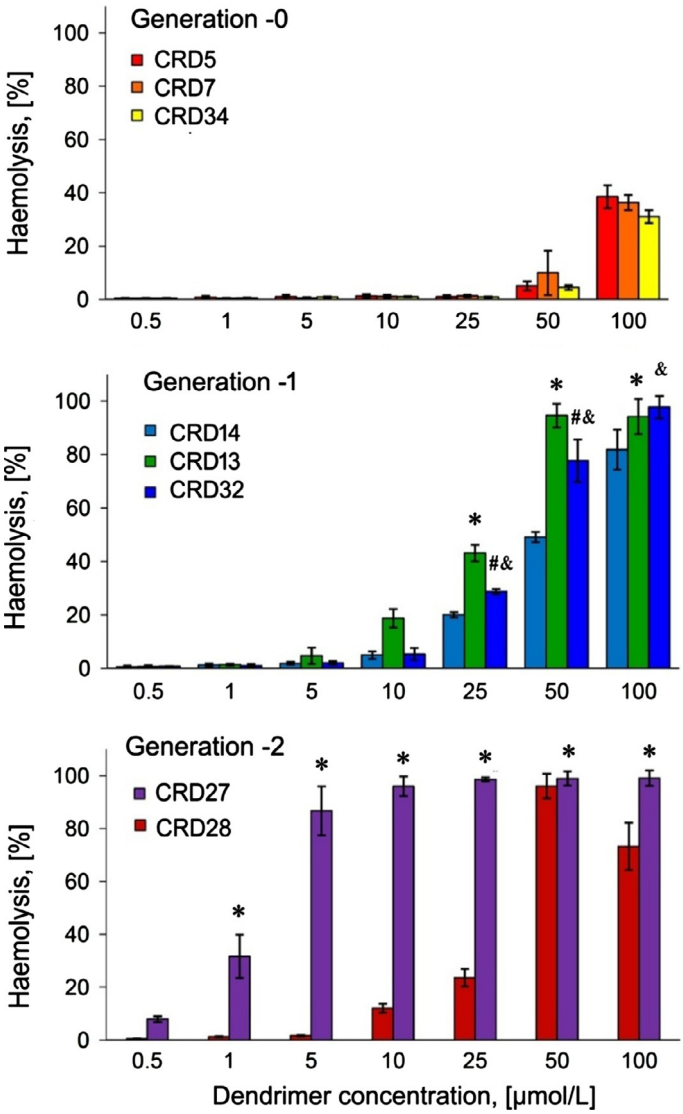


Fig. 2. Hemolysis induced by incubation of red blood cells with ruthenium-terminated carbosilane dendrimers of generation 0 (**CRD7, CRD5, CRD34**), 1 (**CRD13, CRD14, CRD32**) and 2(**CRD27, CRD28**) at 0.5 to −100 μmol/L. Incubation time 24 h, 2% of haematocrit in PBS, pH 7.4, at 22 °C. Results are mean ± SD, n = 6. *P < 0.05 CRD5-CRD7-CRD14 and CRD13-CRD27-CRD28; #P < 0.05 of CRD7-CRD34-CRD13 and CRD32; &P < 0.05 of CRD5-CRD34-CRD14 and CRD32.

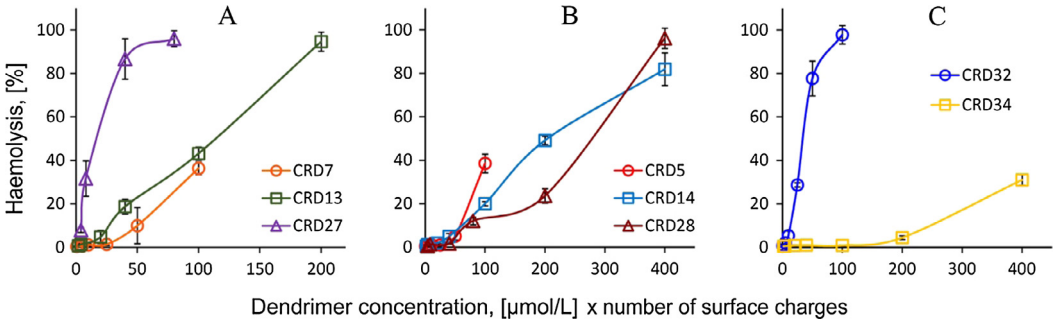


Fig. 3. Dependence of erythrocyte haemolysis on concentration of CRD recalculated on the number of surface charges. The coordination of ruthenium: (A) pyridine-imine groups, **CRD7, CRD13,CRD27**; (B) imine-pyridine groups **CDR5, CRD14,CRD28;** (C) —NH_2 groups **CDR34,CRD32**. ○ – generation 0; □ – generation 1; Δ – generation 2. Incubation 24 h, 2% of haematocrit in PBS, pH 7.4, 22 °C. Results represent as mean ± standard deviation (SD), n = 6.

cationic end groups allow to contact and penetrate cell membrane that can be successfully used in delivery of drugs or genes into the cells.

2.3. Determination of dendrimers cytotoxicity

We estimated the impact of CRD dendrimers on the viability of normal, B14 and cancer, HL-60 cells (Fig. 4). The viability of both kindscells decreased with increasing concentrations of dendrimers. Results show that higher CRD generations were more cytotoxic. Studies indicated no changes in viability of normal (B14) cells in the presence of **CRD5** and **CRD34**

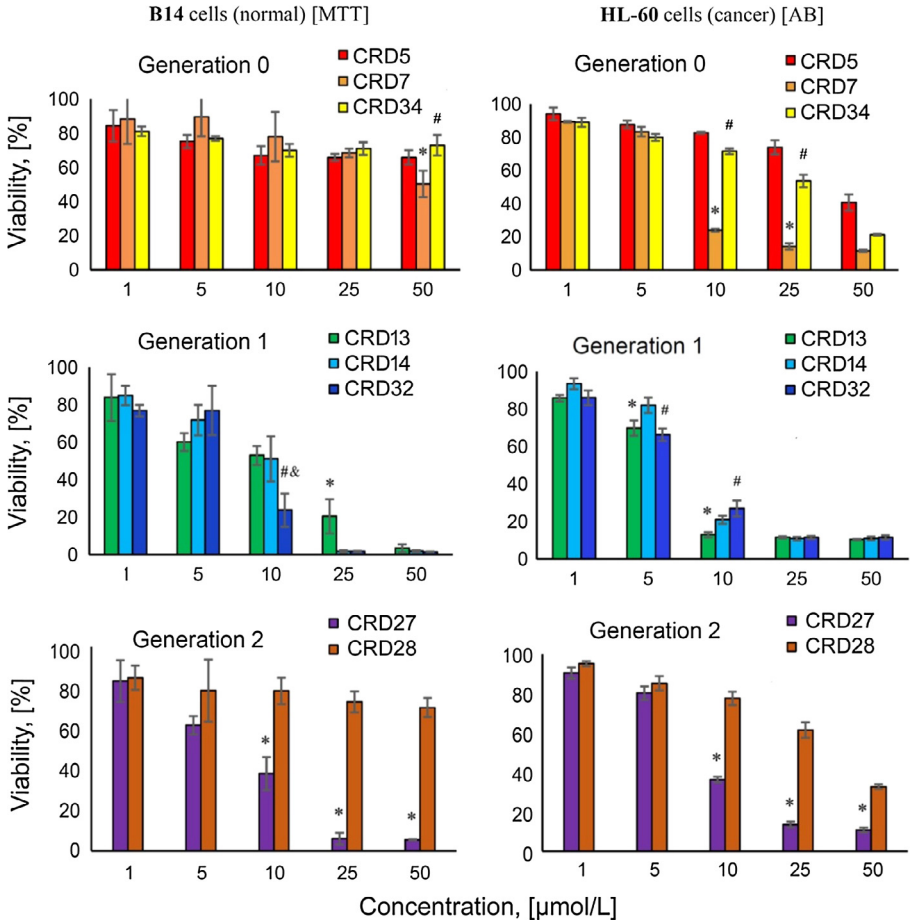


Fig. 4. Viability of B14 (left panels) and HL-60 cells (right panels) after 24 h exposure of ruthenium terminated carbosilane dendrimers at concentration range 1–50 µmol/L. Results represent as mean ± standard deviation (SD). *P < 0.05 CRD5-CRD7-CRD14 and CRD13-CRD27-CRD28; #P < 0.05 of CRD7-CRD34-CRD13 and CRD32; &P < 0.05 of CRD5-CRD34-CRD14 and CRD32.

(Generation - 0) in a concentration range from 1 to 50 μmol/L. In contrast the same **CRD34** significantly decreased the viability of cancer (HL-60) cells up to 53.7% (25 μmol/L) and to 21.2% (50 μmol/L) vs control (Fig. 4, left panel). **CRD5** at the concentration of 50 μmol/L caused the reduction of the number of living cancer cells to 40.7%. At the presence in cell suspension of **CRD7** at the concentrations 25 μmol/L and 50 μmol/L viability of the cells decreased to 68.5% and 50.3% respectively for B-14 cell line (Fig. 4, right panel.) and 14.2% and 11.5% respectively for HL-60 cell line (Fig. 4 left panel).

CRD dendrimers of generation-1 at the concentrations from 1 to 5 μmol/L were not cytotoxic to both normal and cancer cell lines. **CRD**s**14** and **32** at the concentration of 10 μmol/L caused decreasing of the number of living cells to 20.9% and 27% (HL-60) and 51% and 23.7% (B-14) respectively (Fig. 4). These dendrimers added to cells in the concentrations 25 μmol/L and higher, reduced cells viability up to 10–11% for HL-60 and to 1–2% for B-14 cells. **CRD13** applied at concentration 10 μmol/L decreased number of living B-14cells up to 52.9% and up to 20.4% at concentration of 25 μmol/L, at concentration 50 μmol/L up to 3.4%. The same dendrimer added to suspension of HL-60 at the concentration of 10 μmol/L reduced living cells number up to ~12% (Fig. 4).

Addition to the cell suspension of **CRD27** (second generation)at the concentration 10 μmol/L caused decreasing the number of living cells up to ~37% for both lines. Increased concentration of **CRD27** up to 25 μmol/L reduced cell viability up to 10–13% for HL-60 and to 5–6% for B-14 cells. There were no changes in the viability of B14 cells in presence of **CRD28** in a concentration range from 1 μmol/L to 50 μmol/L. In case of HL-60 cell line the addition of **CRD28** at the concentration 50 μmol/L decreased the viability of HL-60 cells up to 32.9% (Fig. 4). The IC_{50} values of each dendrimer for both cell lines considered in this study are summarised in Table 2.

Dendrimer toxicity has previously been described in terms of their generation, size, surface charge and concentration [27,29,35]. The impact of ruthenium-terminated carbosilane dendrimers in our study of the viability on numerous cancer cell lines was also previously assessed [22]. All compounds were cytotoxic, affecting cancer cells in different ways. **CRD7** was significantly toxic to cancer the cell lines MCF-7, HeLa, cervical colon and HT-29, but these dendrimers under the same conditions were 10 times less toxic to MDA-MB-231 cells. CRD dendrimers of generation 1 and 2 were equally toxic to both cancer and normal (HEK-293T) cells, although since the HEK-293T cell line is immortalized, this may result in its higher sensitivity to dendrimers [22].

From Fig. 4, the first important conclusion is that the most promising results were with all the dendrimers of generation 0. For these 3 dendrimers, the significant difference was in their action on normal cells (B14) compared with cancer cells (HL60), the latter showing greater cytotoxicity. This might be due to the difference in the proliferation rate of the cell lines; B14 is a short-term culture of normal Chinese hamster fibroblasts that doubles quickly in ~15 h [36], whereas HL-60 (human promyelocytic leukemia) cells have a doubling time of ~34 h with a cell cycle duration of 24.3 ± 4.1 h [37]. It is known that intracellular ruthenium can slow down DNA and RNA synthesis thereby blocking proliferation [3,38], and ultimately can initiate the apoptotic pathway [7,11]. Cytotoxicity of ruthenium-containing compounds on human lung carcinoma [8,38] and breast cancer (4T1) has been reported [39]. Similar toxic effects of ruthenium compounds on the viability of the hepatocellular carcinoma cells (HCC) and normal human liver cells (L02) were also noted. Ruthenium has been shown to be more toxic to tumour cell lines than normal lines.

The second important conclusion is generation-dependent effect, the higher generations of dendrimers the greater effect because of faster entrance of corresponding dendrimers into cells (generation-dependent hemolytic and cytotoxic effects). The data can explain the difference in the effect of dendrimers of generation 0 on normal (slow proliferating) and cancer (fast proliferating) cell lines: dendrimers of this generation poorly enter cells and can be introduced during parts of the cell cycle. The third important conclusion is an absence of coordination mode dependence on cytotoxicity due to the fact that all the three generation 0 ruthenium metallodendrimers induced similar hemolytic and cytotoxic effects.

Therefore, the increased toxicity of the compounds on cancer cells compared with normal cells, can be caused by ruthenium itself as well as its association with ruthenium carbosilane dendrimers.

3. Conclusions

First, contrary to behaviour seen in adherent cells in which the first generation metallodendrimers were the most active systems [22], generation 0 metallodendrimers are the most effective drugs on suspension cells (HL60). A significant differ-

Table 2
IC_{50}, [μM] of carbosilane ruthenium terminated dendrimers in B14 (normal) and Hl-60 (cancer) cell lines.

Dendrimer	Generation	Surface groups	B 14, IC_{50}, [μM]	SD[a]	HL-60 IC_{50}, [μM]	SD[a]
CRD5	0	sg1	53.78	±4.92	42.18	±3.15
CRD7	0	sg1	35.13	±10.16	12.53	±1.39
CRD34	0	sg1	64.82	±3.55	29.59	±2.16
CRD13	1	sg4	14.04	±6.71	11.57	±1.63
CRD14	1	sg4	12.10	±5.27	12.05	±2.23
CRD32	1	sg4	11.18	±5.12	12.01	±2.70
CRD27	2	sg8	11.81	±5.21	12.94	±2.07
CRD28	2	sg8	65.03	±7.48	29.81	±2.69

[a] Means ± SD from 3 independent experiments.

ence in their effect on normal and cancer cell lines was found. Second, increase of generation leads both to increased cytotoxicity of Ru-dendrimers and the levelling off of any difference in their action on normal and cancer cells. Third, the coordination mode of ruthenium to dendrimer scaffold did not significantly affect their cytotoxicity. Presented results suggest that new synthesized ruthenium terminated dendrimers can be considered as new tool in therapies of reducing cancer cells proliferation.

4. Experimental section

4.1. Carbosilane ruthenium terminated dendrimers (CRD)

The main characteristics and synthesis of CRDs have been described elsewhere [22], their structures being shown in Scheme 1.

4.2. Transmission Electron Microscopy (TEM)

The morphology of ruthenium terminated dendrimers was examined by TEM, images being obtained using a JEOL-1010 (JEOL Ltd., Tokyo, Japan) electron microscope. Ten microliters of each sample at 1 mmol/L in Na-phosphate buffer 10 mmol/L, pH 7, were dropped on 200-mesh carbon-coated copper grids10 min. at room temperature. The samples were stained with saturated solution of uranyl acetate (Sigma) for 20 min, washed in demineralized water and dried at room temperature. The colour of the microphotographs has been reversed and the images been sharpened for greater contrast.

4.3. Zeta potential

Zeta potential was estimated using a Photon Correlation spectrometer Zetasizer Nano ZS, Malvern Instruments (UK). The values were calculated directly from the Helmholtz-Smoluchowski equation using Malvern software. Experiments used Na-phosphate buffer, 10 mmol/L, pH 7.4,at the room temperature. From 9 to 12 measurements of zeta potential were collected and averaged for each sample.

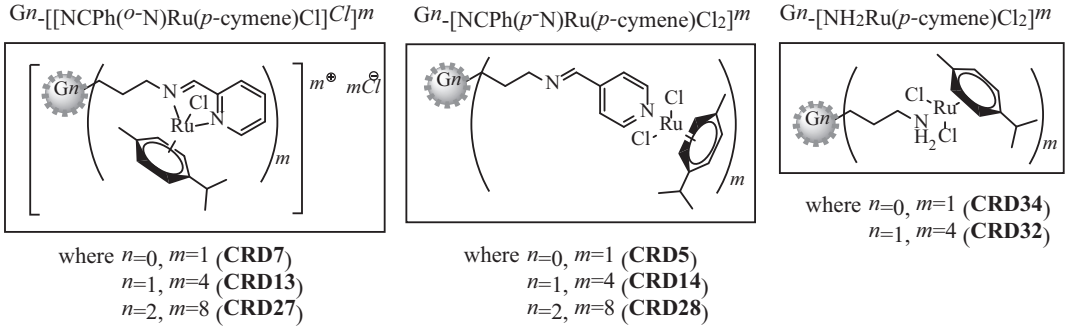
4.4. Measurement of particle size

The particle size and size distribution of dendrimers were measured with a Malvern Zeta-Sizer Nano-ZS (UK) spectrometer by the dynamic light scattering (DLS) technique. The wavelength was set at 633 nm with a detection angle of 90°, refraction factor was 1.33. The samples were prepared in Na-phosphate buffer 10 mmol/L, pH 7.4 filtered twice through 0.22 mm filter paper prior to use. Particle size of dendrimers was measured from the average of 9 × 3 cycles at 25 °C. Malvern software was used to analyse the data of nanoparticle size.

4.5. Haemotoxicity

Blood from healthy donors was purchased from Central Blood Bank, Lodz. Anticoagulated blood was centrifuged and washed 3times with PBS, pH7.4. Erythrocytes were used immediately after isolation. Dendrimers at 0.5–100 μmol/l were added to red blood cells at a 2% haematocrit level and incubated at 37 °C for 24 h with shaking. The% of haemolysis was calculated as follows:

$$\boldsymbol{H}(\%) = (\boldsymbol{A_{pb}}\ 540\ \text{nm}/\boldsymbol{A_{water}}\ 540\ \text{nm}) \times 100\%$$



Scheme 1. Structure of 3 families of water/DMSO soluble newly synthesized ruthenium terminated carbosilane dendrimers. The coordination of ruthenium is through pyridine-imine groups (**CRD7, CRD13** and **CRD27**), imine-pyridine groups (**CDR5, CRD14** and **CRD28**) and —NH_2 groups (**CDR34** and **CRD32**).

where $\boldsymbol{H}$ (%) is the percentage of haemolysis of the erythrocytes, $\boldsymbol{A_{pb}}$ 540 nm is the absorbance of the erythrocytes samples incubated with dendrimers, $\boldsymbol{A_{water}}$ 540 nm is the absorbance of the sample after complete haemolysis with water (100%).

4.6. Cells

Two cell lines, HL-60 (suspension promyelocytic human leukemia) and B14 (Chinese hamster fibroblasts), were used to test cytotoxicity. B14 cells were grown in DMEM-Glutamax (Gibco) with 10% heat-inactivated FBS (HyClone). HL-60 cells were grown in RPMI-1640 (Gibco) with 10% heat-inactivated FBS (HyClone) under standard conditions at 37 °C.

4.7. Cytotoxicity

The cytotoxicity of dendrimers was assessed by the MTT assay (B14 cell line) and the AB assay (HL-60 cell line). After 24 h treatment the medium was refreshed. Viability was calculated from the formula:

$$\%\ \text{viability} = (\mathbf{A}/\mathbf{A_c}) \times 100\%$$

where $\boldsymbol{A}$ represents the absorbance of the sample, $\boldsymbol{A_c}$ is the absorbance of control cells.

4.8. Statistical analysis

For hemotoxicity testing, results were obtained from a minimum of 3 independent experiments and are presented as mean ± standard deviation (SD). For the MTT test, the results were given as mean ± SD.

Student's *t*-test was used for statistical analysis. Significance was accepted at $p < 0.05$.

∗ – significant difference between **CRD5** and **CRD7**; **CRD14** and **CRD13**; **CRD27** and **CRD28**;
– significant difference between **CRD7** and **CRD34**; **CRD13** and **CRD32**;
& – significant difference between **CRD5** and **CRD34**; **CRD14** and **CRD32**.

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