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Boronated Derivatives of Protohemin IX with L-Amino Acids as Potential Anticancer Agents

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Abstract—New water-soluble conjugates of protohemin IX with an anionic 1-carba-*closo*-dodecaborate polyhedron and L-amino acids have been synthesized. In these compounds, the amino acid residues are bound to the porphyrin ring through the amide or ester bond. The new water-soluble amino acid derivatives of boronated protohemin IX show high antitumor activity for human tumor cell lines.

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The ability of porphyrins to selectively accumulate and persist for a long time in tumor cells is behind the interest in the synthesis of boronated derivatives of natural and synthetic analogues of porphyrins for their testing in boron neutron capture therapy (BNCT) for cancer [1]. BNCT is a promising method of treatment of human tumors. The method is based on the nuclear reaction of the stable isotope ^{10}B with thermal neutrons. The capture reaction $^{10}B(n, \alpha)^7Li$ yields highly energetic helium nuclei and ^7Li recoil nuclei, their combined path lengths being comparable with the cell diameter (10 μm), which leads to a selective radiation effect at the cell level [2].

Despite the high efficiency of some synthesized carboranylporphyrins, the problem of decreasing their toxicity is still acute since, upon introduction into the body, most available preparations led to the development of undesirable effects, such as blood clot formation and a decrease in erythrocyte concentration, even before the necessary therapeutic concentration of boron in the tumor had been achieved [3].

Preparations should meet the following requirements: (i) good water solubility, (ii) low dark toxicity for nontumor cells, and (iii) chemical stability in vivo and predominant accumulation in tumors (the tumor : brain and tumor : blood concentration gradients should be > 4 : 1). We assumed that the main chemical modification, conjugates of natural protohemin IX with anionic 1-carba-*closo*-dodecaborate (monocarborane) and natural amino acids located at the periphery of the macrocycle, will meet all three conditions. The introduction of an amino acid should optimize the selectivity of the uptake of a compound in rapidly proliferating cells and increase its solubility in water. Previously, boronated porphyrins with neutral and anionic boron polyhedra were synthesized. Among the synthesized compounds, water-soluble 1,3,5,8-tetramethyl-2,4-divinyl-6(7)-[2-(*closo*-monocarboran-1"-yl)methoxycarbonylethyl]-7(6)-(2'-carboxyethyl)porphyrinatoiron(III) (**1**) turned out to be nontoxic in the range 100 mg/kg and an active phototoxic agent in vivo [4]. Study of the intracellular distribution of compound **1** showed that it accumulates in cytoplasm and does not bind to double-strand DNA.

To optimize the anticancer properties of compound **1**, methods were developed for conjugation of L-amino acids to the carboxyl group of boronated porphyrin **1**, which exhibits biological activity in vivo.

Two types of amino acid derivatives based on porphyrin **1** were obtained, in which L-amino acids are bound to the free carboxyl group of monocarboranylporphyirin **1** either through the amide bond or through the ester bond.

Derivatives of the first type were obtained by the reaction of serine, valine, phenylalanine, tyrosine, and tryptophan methyl esters. The porphyrin carboxyl groups were activated by Boc_2O (Scheme 1). The resulting compounds are dark red crystals, which are soluble in CH_2Cl_2, $CHCl_3$, MeOH, and THF.

Compounds of the other type were obtained by the reaction of monocarboranylporphyrin with oxazaborolidine derivatives of L-serine (**7**) and L-threonine (**8**), which have a free hydroxyl group (Scheme 2). Activation was carried out analogously to the method presented in Scheme 1. Intermediate compounds were not isolated since they are readily hydrolyzed to give end products. These porphyrins are of interest since their amino acid moiety contains free amino and hydroxyl groups, which enhances the hydrophilicity of these compounds. The resulting compounds are dark red crystals, which are soluble in CH_2Cl_2, $CHCl_3$, MeOH,

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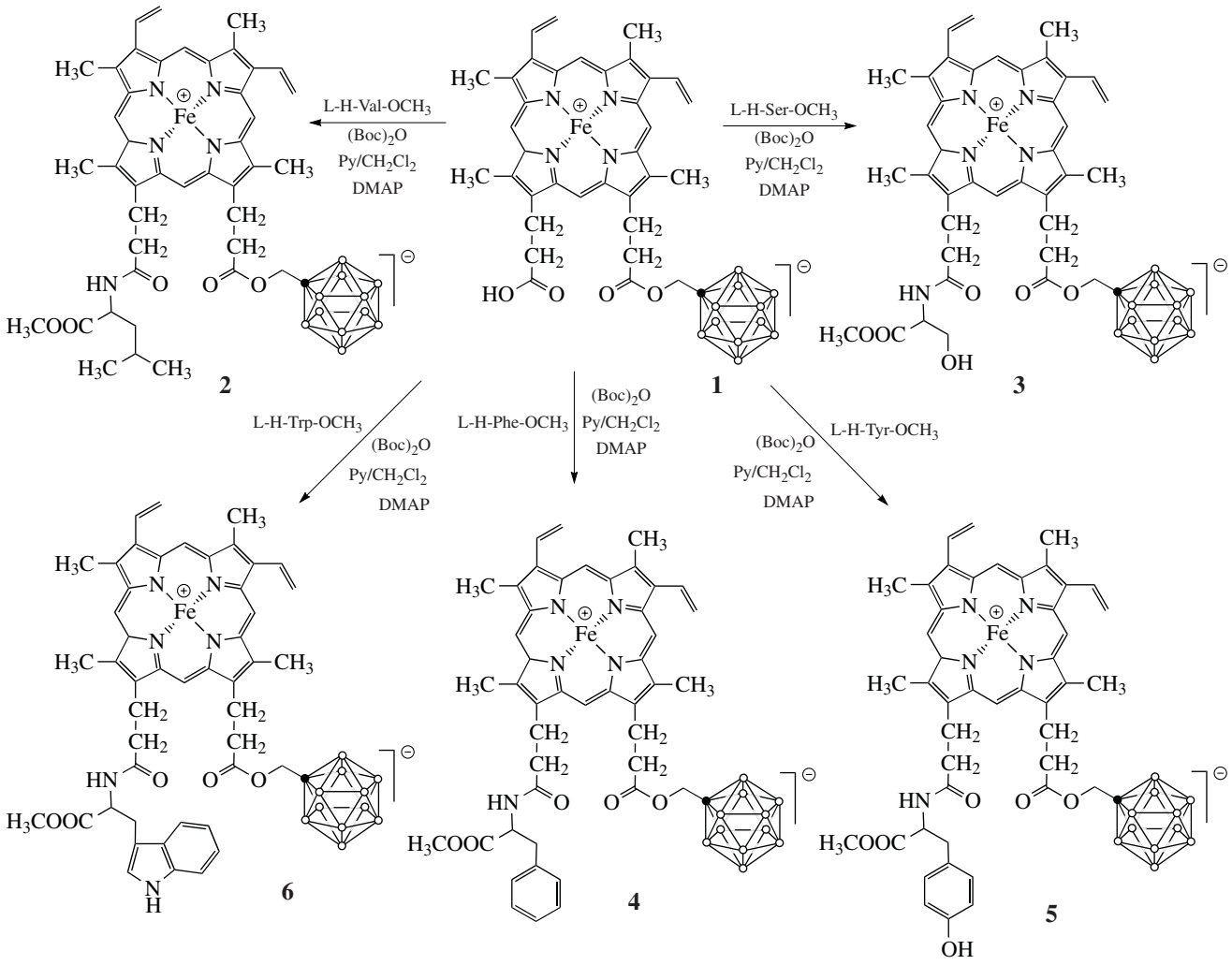
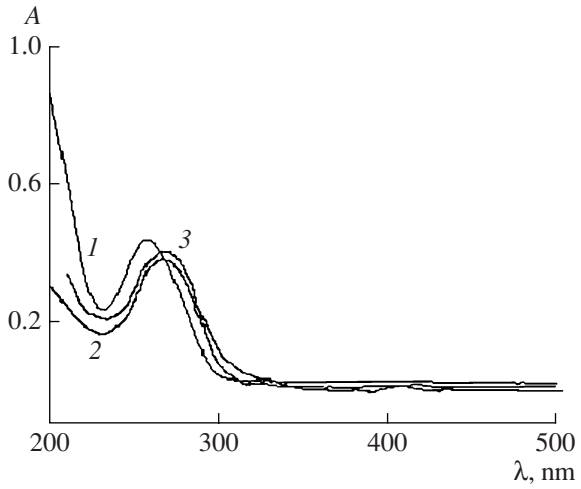


Scheme 1.

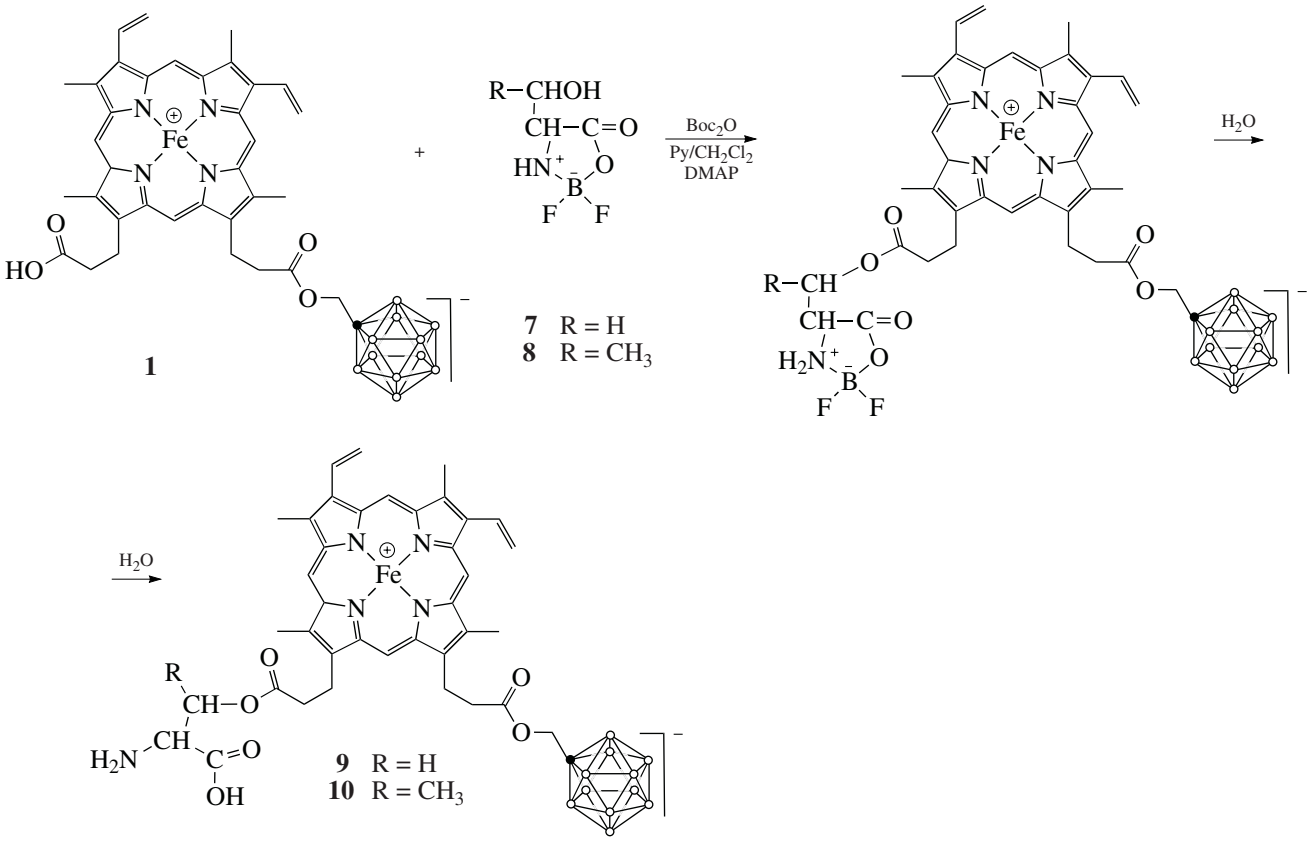
and THF. Their structure was confirmed by electronic absorption and IR spectroscopy and mass spectrometry.

A series of tests for anticancer activity were undertaken. Spectrophotometry showed that compounds **2** and **3** interact with DNA, whereas no such interaction was observed for initial monocarboranylporphyrin **1**. The formation of the molecular complex was judged from the changes in the spectrum of the compound induced by the introduction of DNA (figure). To exclude the contribution of DNA absorption to the spectrum of the reaction mixture, an aqueous DNA solution of the same concentration as that of the reaction mixture was used as the reference solution.

The antitumor activities of the compounds with respect to the K562 cell line (leukemia) were compared. Cells were incubated with 0–50 μM of each agent for 72 h and then reduced by 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan cells (MTT test). The test showed that serine-containing derivative **3** was the



Absorption spectra of aqueous solutions of (*1*) DNA, (*2*) DNA incubated with porphyrin **2**, and (*3*) DNA incubated with porphyrin **3**.



Scheme 2.

most toxic (IC_{50} ~ 5 μM). The analogous valine-containing derivative **2** was considerably less active (IC_{50} ~ 35 μM), whereas carboranylporphyrins with aromatic amino acids **4–6** were inert. The serine carboranylporphyrin exhibited the same activity with respect to the cell lines MCF-7 (human breast carcinoma), CaOv (ovarian carcinoma), and HCT116 (colon adenocarcinoma).

Comparison of the cytotoxicity of monocarboranylporphyrin **1** and its serine derivative **3** showed that the introduction of serine is responsible for the cytotoxicity of the complexes.

Based on the data obtained, we studied the ability of the serine-containing carboranylporphyrin to overcome the multidrug resistance of different tumor cell lines. Two molecular determinants were used: the transmembrane protein P-glycoprotein and the deletion of proapoptotic protein p53. These data showed that new amino acid derivatives of the boronated porphyrin are not transported by P-glycoprotein. In addition, serine-containing carboranylporphyrin causes the death of human colon cancer cells (HCT116) with intact (wild-type) p53 in the same concentrations as in the case of isogenic cells with deletion of both alleles (HCT116p53). Compound **3** was active even at low micromolar concentrations. To elucidate the mechanisms of cytotoxicity of serine carboranylporphyrin, we checked whether this compound induces the production of free oxygen species. *N*-Acetylcysteine (NAC) was used as the chelator of oxygen free radicals. However, the presence of NAC had no effect on the toxicity of the serine-containing carboranylporphyrin. We arrived at the conclusion that oxygen radicals are not involved in the cell death under the action of the serine-containing carboranylporphyrin. These data allow us to assume that the cells stable to the "oxygen explosion" will be sensitive to the serine-containing carboranylporphyrin.

The fragmentation of genomic DNA is an important sign of apoptosis. We were interested in whether the cell death is related to DNA fragmentation caused by the serine-containing carboranylporphyrin. K562 cells treated with the serine-containing carboranylporphyrin were lysed in a buffer containing propidium iodide for binding this fluorochrome to DNA and analyzed in a flow cytometer. The amount of destroyed DNA depended on the dose and time. Within 24 h of the exposure of cells to 10 μM serine carboranylporphyrin, more than 40% of DNA was destroyed, and within 48 h, more than 60%. These results showed that serine carboranylporphyrin can cause cell death by the mechanism of apoptosis accompanied by loss of DNA integrity.

Thus, our findings show that the amino acid derivatives of boronated protohemin containing a water-solu-

ble monocarborane exhibit properties necessary for creating both independent antitumor drugs and drugs for BNCT.

EXPERIMENTAL

The purity of compounds was monitored by TLC (Silufol plates (Czech Republic), chloroform–methanol (9 : 1) system). The IR spectra were recorded as tablets with hexachlorobutadiene on a Carl Zeiss Specord M-82 spectrophotometer. The compounds were purified chromatographically on columns packed with Merck L silica gel (0.040–0.08) (elution with chloroform–methanol, 9 : 1).

General procedure of synthesis of conjugates of 3–6 in which L-amino acids are bound to monocarboranylporphyrin through the amide bond. To a suspension of 0.195 mmol of the corresponding L-amino acid methyl ester hydrochloride in 2 mL of CH_2Cl_2, 0.015 mL of Et_3N was added, and the mixture was stirred at room temperature for 1 h. The solvents were removed in vacuo. Dry ether was added to the residue, and $Et_3N \cdot HCl$ was separated by filtration.

To a solution of 50 mg (0.065 mmol) of porphyrin **1** in a mixture of 4 mL of methylene chloride and 4 mL of pyridine cooled to 0°C, 60 mg (0.275 mmol) of di-*tert*-butylpyrocarbonate was added, and the mixture was stirred at this temperature for 15 min. Then, a solution of the L-amino acid methyl ester in 2 mL of methylene chloride was added, and 10 mg of *N*,*N*'-dimethylaminopyridine was introduced 5 min later. The resulting mixture was stirred for 2 h at 20°C. The solvents were removed in vacuo. The product was purified by column chromatography.

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2'-(*closo*-monocarboran-1-yl)methoxycarbonylethyl]-7(6)-[2-N^{α}(O^{α}-methyl-L-valino)carbonylethyl]porphyrinatoiron(III) (2). The amino acid component was 33 mg of L-valine methyl ester hydrochloride. The yield was 34 mg (59%), $R_f = 0.69$. UV (CH_2Cl_2), λ_{max}, nm ($\varepsilon \times 10^{-3}$): 385.8 (38.30), 511.0 (4.09), 542.2 (3.77), 643.2 (1.83). IR, ν, cm^{-1}: 3383 (NH), 2970 (CH), 2545 (BH), 1719 (CO ester), 1657 (CONH), 1623 (C=C), 1554 (amide II). MS, *m*/*z*: 884 [M^+].

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2'-(*closo*-monocarboran-1-yl)methoxycarbonylethyl]-7(6)-[2-N^{α}(O^{α}-methyl-L-serino)carbonylethyl]porphyrinatoiron(III) (3). The amino acid component was 30 mg of L-serine methyl ester hydrochloride. The yield was 26 mg (46%), $R_f = 0.67$. UV ($CHCl_3$), λ_{max}, nm ($\varepsilon \times 10^{-3}$): 397.8 (48.4), 511.0 (5.29), 542.0 (4.68), 644.0 (2.01). IR, ν, cm^{-1}: 3338 (NH, OH), 2947 (CH), 2540 (BH), 1742 (CO ester), 1658 (CONH), 1623 (C=C), 1553 (amide II). MS, *m*/*z*: 872 [M^+].

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2'-(*closo*-monocarboran-1-yl)methoxycarbonylethyl]-7(6)-[2-N^{α}(O^{α}-methyl-L-phenylalanino)carbonylethyl]porphyrinatoiron(III) (4). The amino acid component was 42 mg of L-phenylalanine methyl ester hydrochloride. The yield was 34 mg (59%), $R_f = 0.61$. UV (CH_2Cl_2), λ_{max}, nm ($\varepsilon \times 10^{-3}$): 395.6 (43.8), 511.1 (5.62), 542.3 (4.25), 643.4 (2.64). IR, ν, cm^{-1}: 3389 (NH), 2930 (CH), 2540 (BH), 1725 (CO ester), 1674 (CONH), 1625 (C=C), 1550 (amide II). MS, *m*/*z*: 932 [M^+].

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2'-(*closo*-monocarboran-1-yl)methoxycarbonylethyl]-7(6)-[2-N^{α}(O^{α}-methyl-L-tyrosino)carbonylethyl]porphyrinatoiron(III) (5). The amino acid component was 30 mg of L-tyrosine methyl ester hydrochloride. The yield was 37 mg (60%), $R_f = 0.70$. UV ($CHCl_3$), λ_{max}, nm ($\varepsilon \times 10^{-3}$): 387.9 (45.4), 510.0 (5.27), 542.2 (4.66), 643.6 (2.21). IR, ν, cm^{-1}: 3368 (NH, OH), 2935 (CH), 2545 (BH), 1732 (CO ester), 1668 (CONH), 1623 (C=C), 1552 (amide II). MS, *m*/*z*: 948 [M^+].

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2'-(*closo*-monocarboran-1-yl)methoxycarbonylethyl]-7(6)-[2-N^{α}(O^{α}-methyl-L-tryptophano)carbonylethyl]porphyrinatoiron(III) (6). The amino acid component was 42 mg of L-tryptophan methyl ester hydrochloride. The yield was 41 mg (64.9%), $R_f = 0.55$. UV (CH_2Cl_2), λ_{max}, nm ($\varepsilon \times 10^{-3}$): 393.3 (44.1), 511.1 (5.62), 541.3 (4.23), 642.7 (2.71). IR, ν, cm^{-1}: 3387 (NH), 2915 (CH), 2537 (BH), 1728 (CO ester), 1667 (CONH), 1635 (C=C), 1552 (amide II). MS, *m*/*z*: 971 [M^+].

General procedure of synthesis of conjugates of monosubstituted carboranylporphyrin with amino acids 9 and 10. A sample of 0.45 mmol of lithium salt of serine or sodium salt of threonine was suspended in 3 mL of anhydrous THF, and 0.27 mL of $BF_3 \cdot Et_2O$ was added. The reaction mixture was stirred for 6 h at room temperature and 2 h at 40–45°C. The solution was evaporated in vacuo. To a solution of 50 mg (0.065 mmol) of porphyrin **3** in a mixture of 4 mL of methylene chloride and 4 mL of pyridine cooled to 0°C, 60 mg (0.275 mmol) of di-*tert*-butylpyrocarbonate was added, and the mixture was stirred at this temperature for 15 min and then added to L-amino acid. Five minutes later, 10 mg of *N*,*N*'-dimethylaminopyridine was added, and the mixture was stirred 2 h at 20°C. Then, 5 mL of water was added, and the mixture was stirred for 1 h in the case of serine and for 10 h in the case of threonine. The aqueous layer was separated. The solvents were removed in vacuo. The product was purified by column chromatography.

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2'-(*closo*-monocarboran-1-yl)methoxycarbonylethyl]-7(6)-[2-O^{α}-L-serino)carbonylethyl]porphyrinatoiron(III) (9). The amino acid component was 66 mg of lithium salt of L-serine. The yield was 33 mg (59%), $R_f = 0.17$. UV ($CHCl_3$), λ_{max}, nm ($\varepsilon \times 10^{-3}$): 363.2 (43.79), 511.4 (4.20), 542.0 (2.95), 621.2 (1.02). IR, ν, cm^{-1}: 3700–3100 (OH), 3315 (NH), 2936 (CH), 2540 (BH), 1725 (CO ester), 1623 (C=C). MS, *m*/*z*: 858 [M^+].

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2'-(*closo*-monocarboran-1-yl)methoxycarbonylethyl]-7(6)-[2-O^{α}-L-threonino)carbonylethyl]porphyrinato-

iron(III) (10). The amino acid component was 63 mg of sodium salt of L-threonine. The yield was 30 mg (53%), R_f = 0.69. UV (CH_2Cl_2), λ_{max}, nm ($\varepsilon \times 10^{-3}$): 406.4 (53.32), 538.0 (7.16), 641.2 (2.32). IR, ν, cm^{-1}: 3700–3100 (OH), 3321 (NH), 2925 (CH), 2540 (BH), 1730 (CO ester), 1623 (C=C). MS, *m/z*: 872 [M^+].

Determination of the cytotoxic activity of protohemin IX derivatives for K562 human chronic myelogenous leukemia cells growing in vitro. Cells were cultured in a complete nutrient medium containing RPMI-1640 + DMEM (1 : 1) (PANAEKO, Russia) and 5% FCS (Flow, Great Britain) with addition of 2 mM of glutamine.

Cells in the log phase of growth were harvested from a substrate by versene/trypsin treatment and carefully pipetted until the formation of a single-cell suspension. The suspension was introduced into 50 μL of a buffer (salt solution) and pipetted, and the cells were counted in a Goryaev chamber (the number of cells in 25 large squares was divided by 5, multiplied by 2500, and multiplied by the dilution; the dimension was cells/mL). The cells and medium were placed in a new tube so that the concentration of cells was 15000 cells/mL. In the wells of a 96-well culture plate, 190 μL of the cell suspension was pipetted. Serial dilutions were prepared: 250 μM (10 μL of the initial 1 mM solution was introduced into 90 μL of the medium), 62.5 μM (25 μL of the 250 μM solution was introduced into 75 μL of the medium), and 15.6 μM (25 μL of the 62.5 μM solution was introduced into 75 μL of the medium). Then, 5 and 10 μL from each dilution were pipetted in the wells. In this case, the solvent volumes and changes in the volume of the medium in a well can be ignored. Incubation was carried out for 72 h at 37°C.

Then, 20 μL of an aqueous solution of MTT (5 mg/mL) was introduced into the wells. The cells were incubated for 1 h until the development of a deep dark violet color inside the cells (formazan). The medium was removed, leaving the cells. Then, 100 μL of DMSO was added to the cells, and the mixture was pipetted until it became homogeneous and was then shaken in a shaker for 2 min. The optical density was measured at 540 nm and survival curves were constructed. The OD_{540} of control wells was taken as 100%. The ODs of the wells containing a tested compound in a certain concentration were related to the control OD.

Spectrophotometric study of the interaction of carboranylporphyrins 1, 2, and 3 with DNA. Spectra were recorded on a Carl Zeiss M-40 spectrophotometer. In a quartz cell 1 cm thick, 3 mL of distilled water and 3 μL of DNA were placed (the ultimate DNA concentration was 10 μg/mL). A solution of lyophilized double-stranded calf thymus DNA (10 mg/mL in distilled water) and solutions of the compounds to be tested (10 mM in DMSO) were prepared.

A 3-mL aliquot of distilled water and 3 μL of DNA were placed in a cell (the end DNA concentration was 10 μg/mL). Absorption was measured on a spectrophotometer at a wavelength of 200–900 nm. Then, 3 μL of porphyrin **1** was introduced into a new cell (the end concentration was 10 μM), and absorption was measured on a spectrofluorimeter. A 3-μL portion of the solution of porphyrin **1** was introduced into the cell with the DNA solution, and absorption was measured with respect to a DNA solution of the same concentration. The binding of DNA was judged from the change in the spectrum at 260 nm (the absorption maximum typical of DNA) after subtraction. Analogous measurements were performed with porphyrins **2** and **3**.

DNA binding was monitored by means of a classical intercalator, antitumor antibiotic doxorubicin.

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