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Novel boronated derivatives of 5,10,15,20-tetraphenylporphyrin: Synthesis and toxicity for drug-resistant tumor cells

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Abstract—We have developed the synthesis of boronated porphyrins for potential application in cancer treatment, based on the functional derivatives of 5,10,15,20-tetraphenylporphyrin. Boronated amide derivatives starting from 5,10,15,20-tetra(*p*-aminophenyl)porphyrin and 9-*o*- and 9-*m*-carborane carboxylic acid chlorides were prepared. Also, the reaction of 2-formyl-5,10,15,20-tetraphenylporphyrin with *closo*-C-lithium-*o*- and *m*-carboranes, as well as with *closo*-C-lithium monocarbon carborane, yielded neutral and anionic boronated hydroxy derivatives of 5,10,15,20-tetraphenylporphyrin, respectively. Water-soluble forms of neutral compounds were prepared by deboronation of *closo*-polyhedra with Bu_4NF into *nido*-7,8- and *nido*-7,9-dicarbaundecaborate anions. Monocarbon carborane conjugated with copper (II) complex of 5,10,15,20-tetraphenylporphyrin was active for a variety of tumor cell lines (IC_{50} ~5 μM after 48–72 h of exposure) but was inert for non-malignant fibroblasts at up to 100 μM. At low micromolar concentrations, this compound caused the death of cells that express P-glycoprotein and other mechanisms of resistance to conventional anticancer drugs.

1. Introduction

Porphyrins constitute the prosthetic groups of heme and heme-containing enzymes and are necessary for many vital processes, predominantly, for respiration, by mediating the iron transport and regulating the redox balance.[1–3] The ubiquitous presence of porphyrins in a cell and their physiological importance make them attractive candidates for therapeutic targeting. Porphyrin-type macrocycles serve as the chemical basis for a variety of drugs that are currently used in preclinical and clinical trials.[4] A relatively low toxicity of porphyrins (since they mimic natural metabolites) and entrapment in malignant cells are expectedly the major pharmacological advantages of these compounds. In particular, among natural carriers for the delivery of toxins to the tumor (e.g., amino acids, carbohydrates, nucleotides, nucleosides, phospholipids, and polyamines),[5] porphyrins possess the most remarkable tumorotropic properties, the reason behind their use in binary therapies of cancer. In photodynamic therapy, generation of singlet oxygen by a photosensitizer molecule (e.g., porphyrin) on light irradiation inflicts tumor damage, while the surrounding non-malignant (containing a lower amount of exogenous porphyrin) tissues remain unaffected.[6–8] In a closely related strategy, the boron neutron capture therapy, tumor cells loaded with boronated porphyrin and irradiated with non-toxic neutrons are damaged by a local radioactive process, this damage largely confined to the tumor mass.[9–11] In these modalities, porphyrins play the role of a vehicle that targets the potential killer to the tumor.

Carboranylporphyrins, the complex structures in which the porphyrin ring is linked to the boron-containing polyhedra, can be attractive anticancer agents, given that a high number (potentially dozens) of boron atoms can be delivered to the tumor.[10,12] Although carboranyl-

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porphyrins are mostly investigative drugs for photodynamic and boron neutron capture therapies,[13–15] recent studies strongly suggest that these compounds can be used as single agents for targeting tumor cells. Indeed, carboranes have been reported to down-regulate DNA synthesis, protein kinase C activity, estrogen receptor, and tumor necrosis factor α[5]; these results broaden the potential applicability of carboranyl-substituted porphyrins for mechanism-based anticancer strategies.

This work is a continuation of our studies on boronated porphyrins as anticancer drugs.[16–20] We have developed a new series of carboranylporphyrins, based on the functional derivatives of 5,10,15,20-tetraphenylporphyrin, and various neutral and anionic polyhedral carboranes. Our choice of 5,10,15,20-tetraphenylporphyrin as a basic compound for chemical modifications was guided by the (i) availability of this compound, (ii) suitability for introducing different functional groups, and (iii) ease of obtaining final products with reasonable yield and high purity. In line with our hypothesis[12] that carboranylporphyrins can overcome pleiotropic resistance in cancer cells, in particular, the multidrug resistance mediated by the transmembrane transporter P-glycoprotein (Pgp; ABCB1),[21,22] we found that novel Cu-containing monocarbon carboranyl-substituted 5,10,15,20-tetraphenylporphyrin was highly potent for human malignant cells and their sublines resistant to many xenobiotics including conventional chemotherapeutics.

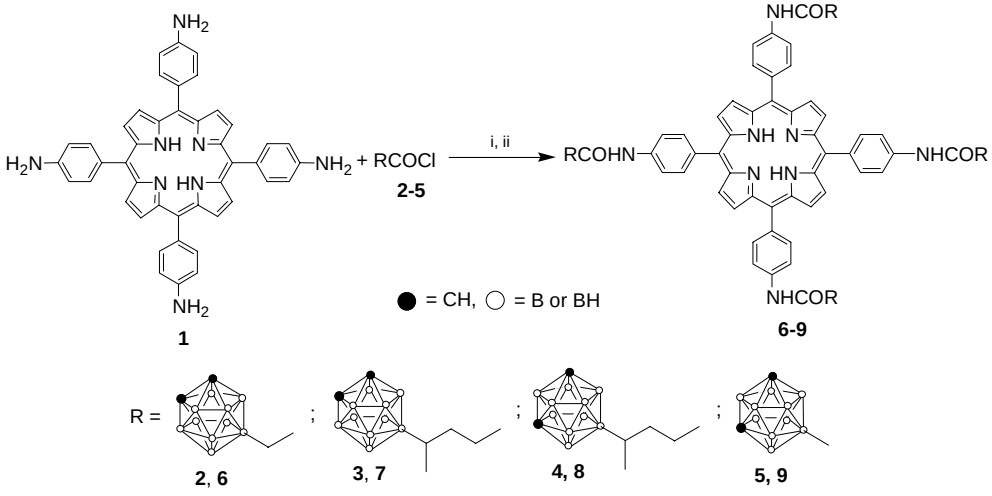
2. Results

2.1. Chemistry

We synthesized two groups of carboranylporphyrins using 5,10,15,20-tetraphenylporphyrin as a starting compound by modifying the phenyl rings and the porphyrin macrocycle. The first group contains carboranylporphyrins in which porphyrin is linked to the carborane moiety via the boron atom of the polyhedron. The second group is represented by carboranylporphyrins in which porphyrin and carborane are connected through the carbon atom of the polyhedron.

The reactions of 5,10,15,20-tetra(*p*-aminophenyl)porphyrin **1** and (*o*-carboran-9-yl)acetic acid chloride (**2**), 4-(*o*-carboran-9-yl)pentanoic (**3**), 4-(*m*-carboran-9-yl)pentanoic acid chlorides (**4**), and (*m*-carboran-9-yl)carboxylic acid chloride (**5**), respectively, yielded carboranylporphyrins **6**–**9** in which the carborane fragment was linked to the porphyrin macrocycle via the amide bond. The acylation of amino groups of porphyrin **1** with acid chlorides **2**–**5** was performed in the CH_2Cl_2/pyridine (1:0.8 v/v) mixture in the presence of Et_3N and catalytic amounts of 4,4′-dimethylaminopyridine (DMAP) under an argon atmosphere (Scheme 1). The amide-linked carboranylporphyrins in which the porphyrin macrocycle was linked to the carbon atom of carborane polyhedron were first synthesized by Rudolph's group[23] for use as catalysts in the reversible multielectron reduction of small molecules, such as O_2 and N_2.

Porphyrin **1** was synthesized, as described,[24] namely, from 5,10,15,20-tetra(*p*-nitrophenyl)porphyrin, followed by the reduction with $SnCl_2$ in HCl. Acid chlorides **2**–**5** were obtained by boiling the corresponding acids[17,25,26] with $SOCl_2$ in benzene solution. The amide porphyrin derivatives **6**–**9** (dark red compounds, 5–45% yield) were purified by column chromatography. In contrast to compounds **2**–**4**, the reaction of porphyrin **1** with acid chloride **5** (in which the COCl group is directly attached to the boron atom of *m*-carborane) resulted in a very low (5%) yield of porphyrin **9**. The main product was a tentatively polymeric violet-red compound, insoluble in organic solvents. It is worth noting that in porphyrins **6**–**9** the carborane substituent is bound to the porphyrin macrocycle through the boron atom of the polyhedra. This makes it possible to modify the characteristics of carboranylporphyrins by introducing various hydrophilic substituents at two unsubstituted CH groups of carborane, thereby decreasing the hydrophobicity of the entire molecule.



Scheme 1. Synthesis of carboranylporphyrins **6**–**9**. Reagents and conditions: (i) CH_2Cl_2–C_5H_5N, Et_3N, DMAP, under Ar, rt, 3 h; (ii) refluxing under Ar, 1 h.

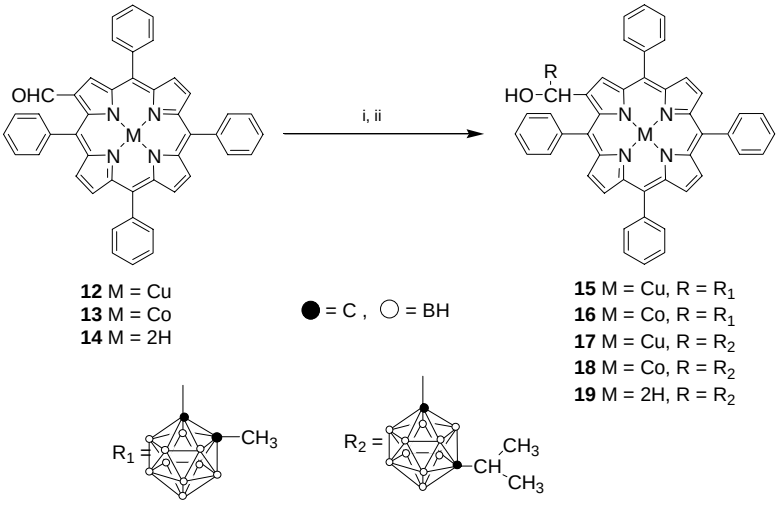
To obtain novel functionally substituted hydrophilic boronated porphyrins, we employed a general approach based on the interaction of carborane carbanions with aldehydes.[27,28] The reaction of 1-lithium-2-methyl-*o*-carborane (**10**) or 1-isopropyl-7-lithium-*m*-carborane (**11**) with the derivatives of 2-formyl-5,10,15,20-tetraphenylporphyrin with copper (II) (**12**), cobalt (II) (**13**), or no metal (**14**)[29] in the coordination sphere of porphyrin macrocycle yielded neutral carboranylporphyrin alcohols **15**–**19**, soluble in organic solvents and insoluble in water (Scheme 2).

The anionic *nido*-carboranylporphyrin alcohols **20**–**22** were synthesized by deboronation[30] of *closo*-analogues **15**, **18**, and **19** with $Bu_4NF{\cdot}2H_2O$ in THF and isolated as tetrabutylammonium salts (Scheme 3).

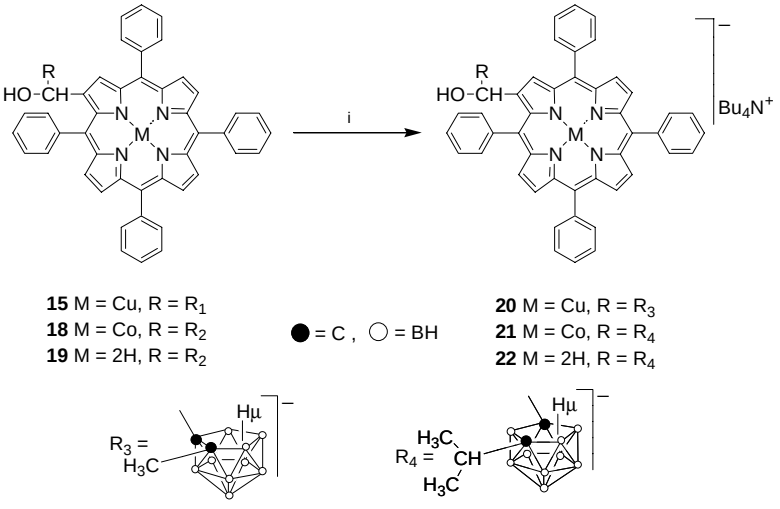
In addition to the above-mentioned strategy, we synthesized a new type of anionic carboranylporphyrin alcohols as synthones for water-soluble analogues. We used a hydrophilic *closo*-monocarbon carborane anion, *closo*-$CB_{11}H_{11}^-$, which is isoelectronic to neutral *closo*-$C_2B_{10}H_{12}$ carboranes. This compound was chosen because (1) monocarbon carborane is stable in air and aqueous media, suggesting its stability in the body; (2) some salts of monocarbon carborane and its hydrophilic derivatives are water soluble on account of the anionic charge; this factor is advantageous over neutral carboranes and makes it possible to obtain hydrophilic boronated porphyrins.

We found that the reaction of 1-lithium-*closo*-monocarbon carboranyl cesium (**23**) with formylporphyrins **12**–**14** in THF resulted in the formation of anionic monocarbon carboranylporphyrin alcohols **24**–**26** in high yield (70–85%) (Scheme 4).

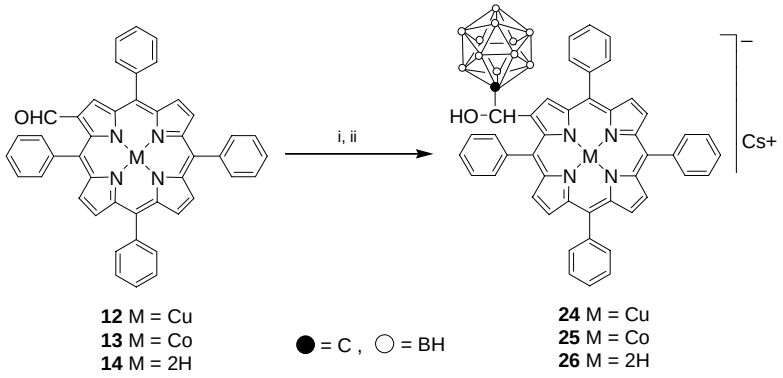
Among the novel monocarbon carborane-substituted porphyrins, only the cesium salt of monocarbon carborane alcohol **26** was, to some extent, soluble in water. Our preliminary data suggest that substitution of cesium cation by sodium or lithium cations results in higher amphiphilicity (not shown).



Scheme 2. Synthesis of carboranylporphyrins **15**–**19**. Reagents and conditions: (i) THF, **10** or **11** under Ar, rt, stirring 2 h; (ii) water, extracting with CH_2Cl_2.



Scheme 3. Synthesis of carboranylporphyrins **20**–**22**. Reagents and conditions: (i) THF, Bu_4NF, refluxing, 2 h.



Scheme 4. Synthesis of carboranylporphyrins **24**–**26**. Reagents and conditions: (i) THF, **23** under Ar, rt, stirring 1 h; (ii) water, extracting with CH_2Cl_2.

All synthesized compounds were isolated by column chromatography as dark red crystals soluble in chloroform, methylene chloride, THF, pyridine, acetone, and acetonitrile. The identity of all compounds was confirmed by mass spectra, electron, and infrared spectra analyses (Table 1). Non-paramagnetic metal free porphyrins (**6**–**8**, **19**, **22**, and **26**) were characterized by ^1H NMR spectra.

2.2. Biological testing

All novel carboranylporphyrins were tested for their ability to kill cultured human cells. The compounds showed differential activity. We found that amide derivatives **6**–**8**, *closo*-alcohols **16**, **18**, **19**, as well as *nido*-alcohols **21**, **22**, demonstrated slight or no cytotoxicity for leukemia and breast carcinoma cell lines (not shown). However, 5,10,15,20-tetraphenylporphyrin caused the death of K562 leukemia cells with $IC_{50} = 52.6 \pm 4.3$ μM, as determined in the MTT test after 72 h of continuous exposure (Fig. 1A). A comparison of the activities of *o*-methyl- and *m*-isopropyl-*closo*- and *nido*-carboranyl-substituted derivatives of Cu-5,10,15,20-tetraphenylporphyrin complex (compounds **15**, **17**, and **20**) and monocarbon carborane-substituted copper (II) complex of 5,10,15,20-tetraphenylporphyrin (**24**) identified compound **24** as the most active ($IC_{50} = 4.6 \pm 2.2$ μM) (Fig. 1A). Next, **24** demonstrated a higher activity for MCF-7 breast carcinoma cell line than the structurally related cobalt (II) salt **25** and metal free **26** carborane derivatives of 5,10,15,20-tetraphenylporphyrin (Fig. 1B). Importantly, **24** was virtually inert for non-malignant human skin fibroblasts at concentrations up to 100 μM within 72 h, whereas this compound potently killed malignant CaOv cells (Fig. 1C). Only at higher concentrations at which **24** formed precipitates in aqueous solutions was this agent toxic to fibroblasts. These experiments provide the evidence that monocarbon carboranes conjugated with the Cu(II) salt of 5,10,15,20-tetraphenylporphyrin could be the basis for further investigation as anticancer agents.

Pleiotropic refractoriness of tumor cells to exogenous stimuli remains a major reason for therapeutic failure. The transmembrane transporter P-glycoprotein (Pgp; ABCB1) frequently mediates the intrinsic (prior to chemotherapy) resistance to apoptosis, as well as multidrug resistance (MDR) acquired in the course of treatment.[31–36] To study the potency of carboranylporphyrins for sublines with Pgp-mediated MDR, we chose **24** because this compound was the most active against parental leukemia and breast cancer cells. We first addressed the role of Pgp in the cytotoxicity of **24** by comparing the survival of K562 cells and the K562i/S9 subline that expresses Pgp without selection.[37,38] The K562i/S9 cells were significantly more resistant than K562 cells to vincristine, the chemotherapeutic drug with high affinity to Pgp (IC_{50}s were 56.1 ± 4.5 nM vs 6.2 ± 2.1 nM, respectively; resistance index 9.0) (data of 3 experiments). In striking contrast, survival of both cell lines in the presence of **24** differed only moderately: the respective (IC_{50}s were 10.5 ± 2.0 μM vs 5.2 ± 1.7 μM; resistance index 2.0). These data suggest that **24** is a weaker substrate of Pgp-mediated transport than vincristine. Nevertheless, inhibition of Pgp function with VER[39] dramatically potentiates the cytotoxicity of **24**. Death of K562i/S9 cells after 24 h of exposure to 4 μM **24** + 20 μM VER (Fig. 2) was almost as pronounced as death of the same cells treated with 16 μM **24** alone (data not shown). Only marginal MTT conversion (Fig. 2) and clearly detectable morphological signs of apoptosis, such as cell shrinkage and nuclear fragmentation, were found in K562i/S9 cells treated with 2 μM **24** + 20 μM VER for 48 h. No significant changes in MTT reduction were detected in these cells after exposure to 4 μM **24** or 20 μM VER alone (Fig. 2), nor did these treatments cause discernible morphological features of cytotoxicity.

To study the potency of **24** for cells that acquired Pgp-mediated MDR during multistep selection with conventional drugs, we compared the cytotoxicity of **24** for MCF-7 cells with that of the MCF-7Dox variant selected for long-term survival in the presence of doxorubicin (DOX). The MCF-7Dox subline displayed Pgp-mediated MDR, as determined by a higher resistance to Pgp-transported chemotherapeutics, such as DOX, vincristine, mitoxantrone, and taxol (Table 2), elevated amount of Pgp, and increased efflux of Pgp-transported fluorescent dye rhodamine 123[40] (Figs. 3A and B). Importantly, prolonged (72 h) exposure to **24** demonstrated a slightly higher (resistance index ~2) survival of MCF-7Dox cells than MCF-7 cells (Fig. 4). However, the combination of 4 μM **24** and VER induced toxic

Table 1. Physico-chemical characteristics of novel carboranylporphyrins

Compound	Infrared spectra, ν, cm^{-1}	Electron spectra ($CHCl_3$), λ_{max}, nm, ($\varepsilon \times 10^{-3}$)	Mass spectra, *m/z*	Elemental analysis		
				Formula	Calc. (%)	Found (%)
6	3318(NH), 3060 (carborane CH), 2598(BH), 1703(CO)	424(150.2), 519(7.9), 561(4.2), 598(2.5), 655(2.1)	$1411.5[M]^+$	$C_{60}H_{82}B_{40}N_8O_4$	C, 51.05; H, 5.85; N, 7.94	C, 50.96; H, 5.92; N, 7.88
7	3318(NH), 3060(carborane CH), 2598(BH), 1703(CO)	424(144.2), 519(8.9), 561(5.0), 598(2.9), 655(2.4)	$1580[M]^+$	$C_{72}H_{106}B_{40}N_8O_4$	C, 54.73; H, 6.76; N, 7.09	C, 54.25; H, 6.67; N, 7.12
8	3318(NH), 3060(carborane CH), 2598(BH), 1703(CO)	424(145.0), 519(8.9), 561(5.1), 598(2.9), 655(2.7)	$1580[M]^+$	$C_{72}H_{106}B_{40}N_8O_4$	C, 54.73; H, 6.76; N, 7.09	C, 54.58; H, 6.78; N, 7.05
9	3318(NH), 3060(carborane CH), 2599(BH), 1700(CO)	424(137.1), 522(9.0), 559(5.8), 598(2.6), 657(2.4)	$1354[M-1]^+$	$C_{56}H_{74}B_{40}N_8O_4$	C, 49.62; H, 5.50; N, 8.27	C, 49.45; H, 5.32; N, 8.12
15	3615(OH), 2983(porphyrin CH), 2576(BH)	419.0(158.5), 544.4(9.3), 583.4(3.8)	$861.5[M-1]^+$	$C_{48}H_{42}B_{10}CuN_4O$	C, 66.84; H, 4.91; N, 6.50	C, 66.80; H, 4.85; N, 6.42
16	3610(OH), 2975(porphyrin CH), 2580(BH)	415.1(127.0), 545.6(22.4), 587.3(12.7)	$858[M-1]^+$	$C_{48}H_{42}B_{10}CoN_4O$	C, 67.20; H, 4.93; N, 6.53	C 67.12; H, 4.80; N, 6.72
17	3605(OH), 2960(porphyrin CH), 2590(BH)	421.2(145.6), 547.0(8.9), 584.3(3.6)	$890.5[M]^+$	$C_{50}H_{46}B_{10}CuN_4O$	C, 67.43; H, 5.21; N, 6.29	C, 67.23; H, 5.44; N, 6.11
18	3630(OH), 2840(porphyrin CH), 2610(BH)	416.0(137.6), 549.4(16.8), 591.0(6.7)	$886[M-1]^+$	$C_{50}H_{46}B_{10}CoN_4O$	C, 67.78; H, 5.23; N, 6.32	C, 67.92; H, 5.64; N, 6.57
19	3602(OH), 2930, 2859(porphyrin CH), 2591(BH)	420.1(197.0), 517.6(27.0), 560.2(11.6), 597.2(10.1), 651.0(7.5).	$831[M]^+$	$C_{50}H_{48}B_{10}N_4O$	C, 72.44; H, 5.84; N, 6.76	C, 72.65; H, 5.59; N, 6.43
20	3610(OH), 2966, 2874(porphyrin CH), 2519(BH)	418.6(120.4), 542.8(6.8)	$1096[M+1]^+$	$C_{64}H_{78}B_9CuN_5O$	C, 70.25; H, 7.19; N, 6.40	C, 69.95; H, 7.37; N, 6.22
21	3612(OH), 2967, 2926(porphyrin CH), 2531(BH)	414.8(111.5), 535.6(19.5)	$1126[M]^+$	$C_{66}H_{82}B_9CoN_5O$	C, 70.93; H, 7.40; N, 6.27	C, 70.52; H, 7.68; N, 6.64.
22	3620(OH), 2963, 2875(porphyrin CH), 2527(BH)	420.0(10.6), 514.2(14.3), 558, 4(8.6), 596.0(5.4), 661.6(3.1)	$1068[M]^+$	$C_{66}H_{84}B_9N_5O$	C, 74.74; H, 7.98; N, 6.60	C, 74.91; H, 7.45; N, 6.23
24	3625(OH), 2960, 2927(porphyrin CH), 2535(BH)	419.4(126.2), 543.8(9.0)	$977.5[M+2]^+$	$C_{46}H_{40}B_{11}CsCuN_4O$	C, 56.37; H, 4.11; N, 5.72	C, 56.78; H, 4.12; N, 5.52
25	3604(OH), 2837(porphyrin CH), 2515(BH)	436.0(154.3), 547.2(10.5)	$978[M-2]^+$	$C_{46}H_{40}B_{11}CsCoN_4O$	C, 56.63; H, 4.13; N, 5.74	C, 56.81; H, 4.05; N, 5.59
26	3612(OH), 2944(porphyrin CH), 2504(BH)	423.6(105.2), 523.8(4.6), 565.2(3.0), 592.0(2.4), 649.2(1.2)	$917.7[M-1]^+$	$C_{46}H_{42}B_{11}CsN_4O$	C, 60.14; H, 4.61; N, 6.10	C, 59.84; H, 4.64; N, 6.18

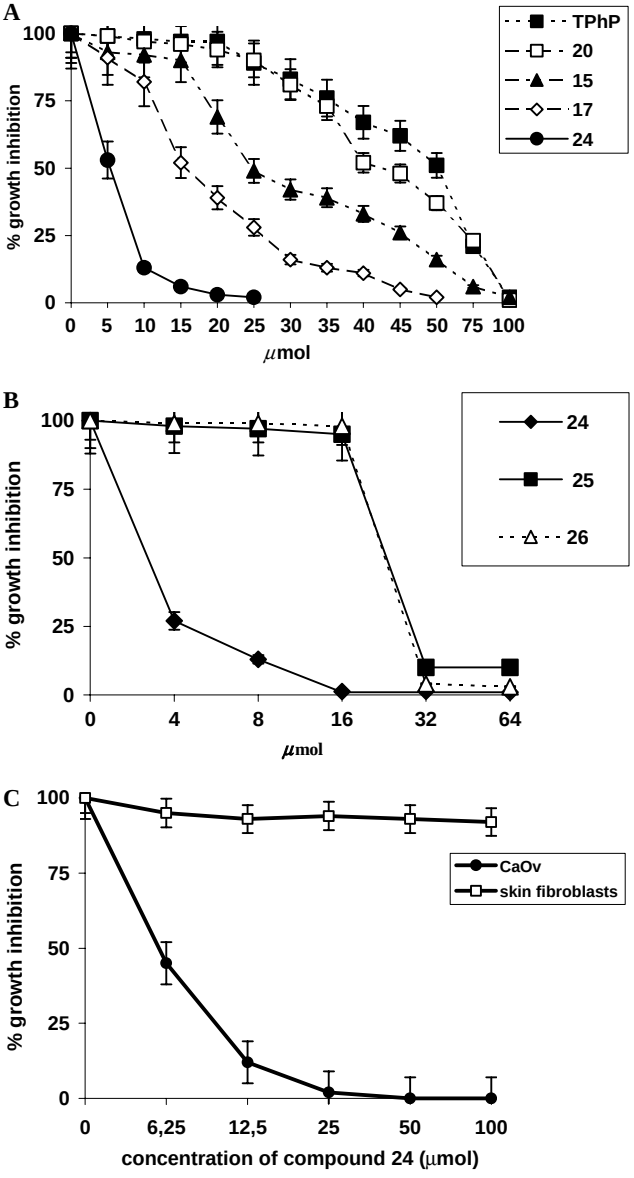


Figure 1. Cytotoxicity of 5,10,15,20-tetraphenylporphyrin (TPhP) and its carborane derivatives. Cell lines: K562 (A), MCF-7 (B), CaOv and skin fibroblasts (C). Cells were treated with indicated compounds for 72 h. Cell viability was determined in MTT test (see Section 4). Cumulative data of five experiments are shown.

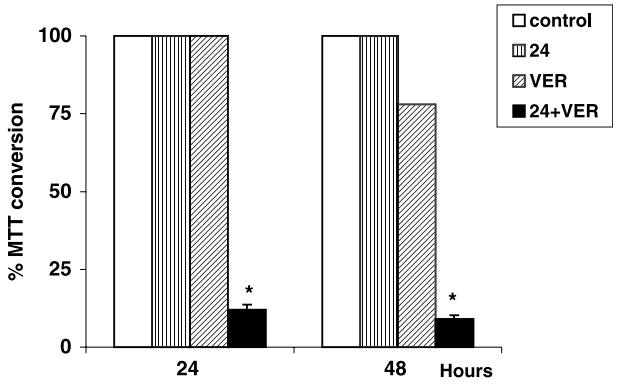


Figure 2. VER potentiates the cytotoxicity of compound **24** for Pgp-expressing leukemia cells. The K562i/S9 cells were treated with 0.05% DMSO (control), 4 μM **24** without or with 20 μM VER for 24 h, or with 2 μM **24** without or with 20 μM VER for 48 h. After treatment MTT solution was added to the cells. Percent MTT conversion was calculated as ratio of OD_{540} of drug treated cells to that of respective control cells (100%). Shown are mean ± SD of three experiments. $^{*}p < 0.01$ compared with each of three groups within the respective time interval.

effects within the initial hours of exposure. Figures 5A–C show that treatment with 4 μM **24** + 20 μM VER caused a time-dependent sparseness of the cell monolayer, with a lesser number of cells adhered to the coverslip by 24 h of exposure (dying cells were washed off before fixation). Cell morphology changed drastically: many cells displayed extended processes, vacuolized cytoplasm, altered structure of the chromatin, and multiple, irregularly shaped nucleolae (Figs. 5D–G). The features of cytotoxicity, such as chromatin compactization, formation of numerous vacuolae-like inclusions, and swelling of the cytoplasm, were discernible as early as after 5 h with 4 μM **24** + 20 μM VER (Figs. 5D and E).

Clinical resistance may not be confined to a Pgp-mediated MDR but is rather multifactorial, with a number of survival mechanisms emerging in the course of tumor progression and adaptation to treatment. The activity of **24** was therefore tested in cells with Pgp-unrelated determinants of drug response. Death, via p53 pathways, has been implicated as an important prerequisite for the efficacy of anticancer therapies.[41] Impairment of p53 functions in cancer cells renders them resistant to DNA damage induced apoptosis.[42] In our experiments, DOX was more potent for a HCT116 colon carcinoma cell line (wild type $p53^{+/+}$) than to its variant with deletion of both alleles of p53 (HCT116p53KO)[43] (data not shown). In contrast, no survival advantage in cells lacking p53 was shown when HCT116 and HCT116p53KO cells were treated with **24** (note similar IC_{50} for both cell lines, Table 3). We next compared the survival of androgen-dependent LNCaP cells and their subline LNCaP-abl selected for long-term androgen ablation.[44] In LNCaP-abl cells, the Akt kinase is constitutively switched on, rendering these cells resistant to induction of apoptosis by the anticancer drug etoposide or by LY294002, an inhibitor of phosphatidylinositol-3′-kinase (PI3K).[44] Compound **24** (at low micromolar concentrations) killed LNCaP and LNCaP-abl cells with equal potency (Table 3). Furthermore, we found no significant differences in the cytotoxicity of **24** for A2780 ovarian carcinoma cells and their cisplatin selected A2780DDP counterparts in which the resistance is conferred by an increased intracellular content of reduced glutathione (GSH)[45,46] (Table 3). Finally, FEMX melanoma cells and their isogenic counterparts resistant to anti-proliferative effect of dexamethasone and hypoxia (due to increased PI3K activity and induction of signal transducer and activator of transcription 3, STAT3)[47] showed similar sensitivity to **24**. Altogether, our data indicate that **24** is capable of circumventing various resistant phenotypes in tumor cells, and this ability is not restricted to a particular cell type.

Table 2. Multidrug resistance in MCF-7Dox subline

Cell line	DOX	Vincristine	Taxol	Mitoxantrone
MCF-7	248 ± 29*	38 ± 11	112 ± 24	138 ± 13
MCF-7Dox	2465 ± 109** (9.9)	5410 ± 135** (14.2)	8120 ± 412** (72.5)	7465 ± 181** (54.1)

* IC_{50} (nM; mean ± SD of three independent experiments) determined in MTT test after incubation for 72 h.
** $p < 0.01$ by Student's t test. In parentheses: indexes of resistance calculated as mean IC_{50} for MCF-7Dox cells divided by mean IC_{50} for MCF-7 cells.

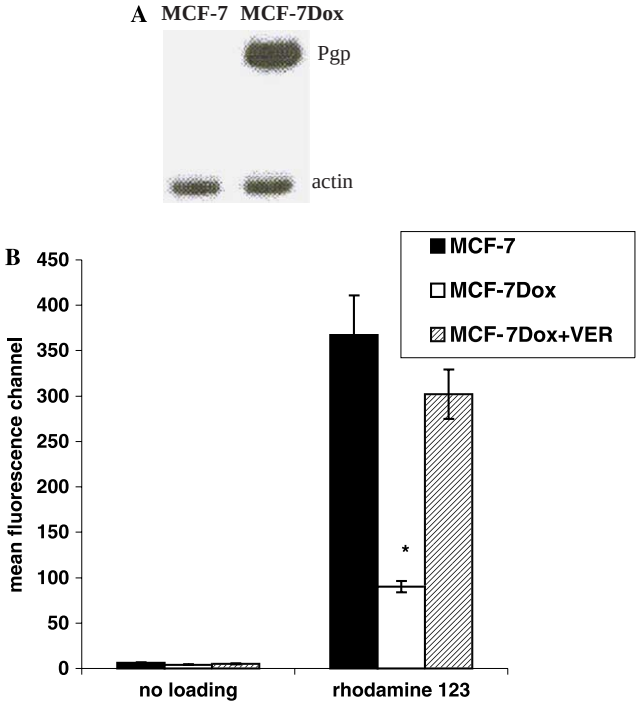


Figure 3. MCF-7Dox cells express functional Pgp. (A) Immunoblot analysis of Pgp in MCF-7 and MCF-7Dox cells. See Section 4 for details. (B) The decreased rhodamine 123 accumulation in MCF-7Dox is reversible by VER. Cells were loaded with 300 nM rhodamine 123 for 1 h at 37 °C; in parallel the MCF-7Dox cells were treated with rhodamine 123 and VER. After treatment cells were washed and analyzed by flow cytometry. Data are mean ± SD of three experiments. *$p < 0.05$ compared with MCF-7 and MCF-7Dox+VER groups.

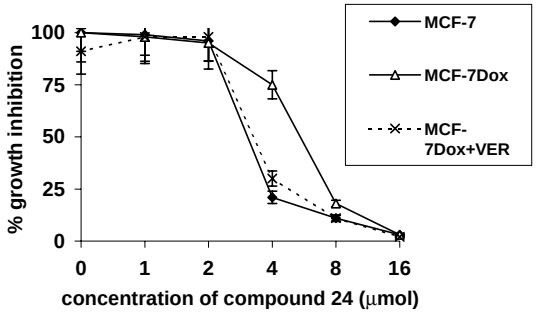


Figure 4. VER sensitizes MCF-7Dox cells to compound **24**. The MCF-7 and MCF-7Dox cells were treated with **24** in the absence or presence of 20 μM VER for 72 h. Cell viability was determined in MTT test. Shown is one experiment out of three with essentially the same results. Each value is mean ± SD of three measurements.

3. Discussion and conclusions

We developed a general method for the synthesis of boron-containing porphyrin alcohols based on the interaction of reactive carborane carbanions with formyl porphyrins. This approach makes it possible to obtain biologically active boronated porphyrins with sterically hindered carborane moieties that can display hydrophobic (neutral *closo*-1,2- and 1,7-carborane substituents) or hydrophilic (7,8- and 7,9-dicarbaundecaborate anions and *closo*-monocarbon carborane anion) properties.

The reasons for differential potency of structurally close carboranylporphyrins (such as **15**, **17**, **20**, **24**–**26**) remain to be elucidated. Apparently, transport through the plasma membrane is a critical factor of cytotoxicity. The compounds **24**–**26** were potent, whereas **7**, the amide derivative that contains four *closo*-carborane polyhedra, was slightly toxic only at >100 μM. However, **7** contains 40 boron atoms and was expected to be more potent than compounds **24**–**26** with 10 boron atoms each. We hypothesize that one reason for the lack of activity of **7** could be attributed to the branched structure of the molecule due to bulky carborane substituents. This might impede the uptake of this compound by the cell. A direct measurement of intracellular drug accumulation/distribution should reveal whether differences in transmembrane transport and entrapment are causatively linked to the higher toxicity of **24** over other carboranylporphyrins, as well as to the potency of **24** for tumor cells but not to non-malignant fibroblasts.

While carboranylporphyrins are currently studied as tentative anticancer agents, anionic monocarbon carboranes, such as $CB_{11}H_{12}^-$, are poorly investigated. We demonstrate that this class of boron-containing porphyrins could be perspective since **24**, the water-soluble Cu(II) salt of monocarboranyl-substituted 5,10,15,20-tetraphenylporphyrin, potently (in the low micromolar range of concentrations) kills human tumor cells otherwise resistant to many apoptotic stimuli. Importantly, in K562 leukemia and MCF-7 breast carcinoma cells some defense mechanisms are operating prior to drug exposure (intrinsic resistance). The K562 cells express anti-apoptotic Bcr-Abl tyrosine kinase and lack functional p53.[48–50] In MCF-7 cells, caspase 3, a critical effector and a point of convergence of many apoptotic pathways, is not expressed due to deletion of a fragment of the *CASP3* gene.[51] Expression of exogenous Pgp (K562i/S9 cells; the genetically defined model of MDR) or establishment of MDR in drug selected MCF-7Dox subline can be considered as models for

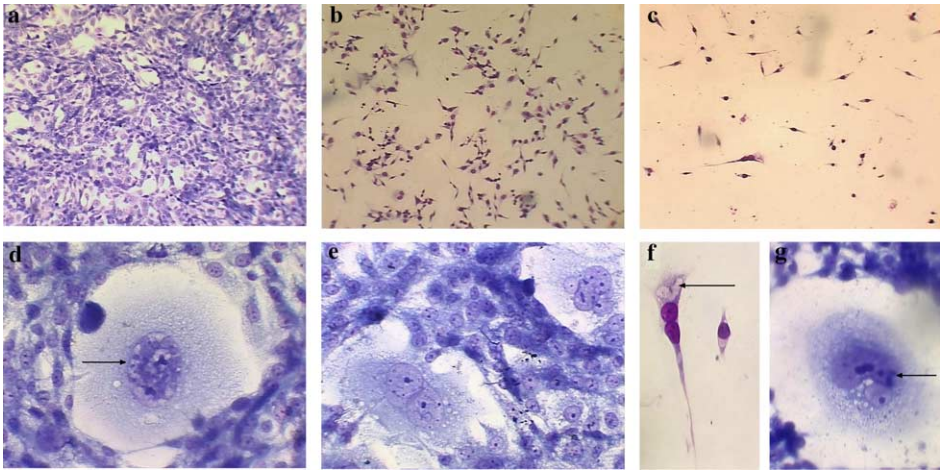


Figure 5. Morphological signs of toxicity of compound **24**. The MCF-7Dox cells grown on coverslips were treated with 0.05% DMSO (control) or 4 μM **24** + 20 μM VER for 5–24 h. Cells were washed with PBS, fixed in methanol, stained with azur and eosin and photographed. (a), control; (b), 5 h; (c), 24 h (original magnification 128×). Note that drug exposure led to fewer cells remaining adhered to the solid support. (d, e) 5 h; (f, g), 24 h (original magnification 400×). Shown are 'giant' cells with dystrophic nuclei (arrow) (d); non-discernible structure of chromatin (e); swelled, abundantly vacuolized cytoplasm with ecto- and endoplasmic areas (d, e); cell elongation and huge abnormal vacuolae-like inclusions (arrow) (f); multiple confluent nucleolae (arrow) (g).

Table 3. Compound **24** is potent for cells with Pgp-unrelated drug resistance

Cell line	Resistance to drugs	Resistance mechanism[Ref]	IC50 (μM) for compound **24**
HCT116			6.5 ± 2.3[*]
HCT116p53KO	DOX	Non-functional p53[**]	6.1 ± 3.0
LNCaP			3.1 ± 1.3
LNCaP-abl	Etoposide, LY294002	Constitutive Akt activation[44]	3.2 ± 1.0
A2780			4.8 ± 1.2
A2780DDP	Cisplatin, carboplatin	Elevated GSH[45,46]	4.4 ± 1.0
FEMX			4.2 ± 1.2
FEMX/D	Dexamethasone	PI3K and STAT3 activation[47]	4.0 ± 1.6

[*] Determined in MTT test after a 72 h exposure; mean ± SD of three independent experiments. No significant differences in IC_{50} were found between the respective parental cell line and its isogenic subline.
[**] Our unpublished results.

acquisition of pleiotropic insensitivity by intrinsically resistant tumor cells. Moreover, the MCF-7Dox cells are highly malignant, as determined by the loss of a pro-apoptotic αvβ3 integrin, markedly activated collagenases and invasiveness of extracellular matrix, the ability to cycle after plating on a non-adhesive support, and resistance to anchorage-dependent apoptosis.[52]

A key prerequisite for overcoming such pleiotropic irresponsiveness should be delivery of the amount of the drug sufficient to activate as many death pathways as possible.[53] This reiterates the importance of transmembrane transport of carboranylporphyrins as a major factor of their anticancer activity (see above). Our results provide evidence that **24** is a relatively poor substrate for Pgp-mediated efflux. Indeed, the cytotoxicity of **24** for Pgp-negative and -positive cells did not differ as substantially as it was in the case of Pgp transported drugs, such as DOX, vincristine, mitoxantrone or taxol, making **24** (and potentially other agents of this class) perspective for circumventing the resistance. This does not underscore the importance of Pgp reversal for augmenting the cytotoxicity of carboranylporphyrins as inhibition of Pgp transport markedly sensitizes Pgp-positive cells to **24**, and the toxic effects of this compound were detectable within the initial hours of exposure to **24** + VER. Therefore, at low micromolar concentrations **24** killed pleiotropically resistant cells, provided a sufficient intracellular concentration of monocarboranylporphyrin is attained. Although Pgp may play a role of general protective mechanism beyond the efflux function,[54] our results suggest that the transmembrane barrier is crucial for the cytotoxicity of carboranylporphyrins. Survival factors, other than the multidrug transporter(s) (such as lack of p53, activated PI3K/Akt pathway, STAT3 signaling cascade, or elevated glutathione), do not confer the resistance to **24**. Intriguingly, these considerations are taken in concert with 'all-or-nothing' mode of cytotoxicity of compounds **24**–**26**, that is, relatively low toxicity at doses below a certain threshold, followed by a dramatic increase of cell death when the concentration raised 2-fold (Figs. 1B and 4). Supposedly, a threshold concentration of active carboranylporphyrins in the cell is a 'point of no return' since this triggers the process(es) that makes death inevitable. On the one hand, such mode of killing should be most appropriate for elimination of tumor cells with multiple mechanisms of resistance. On the other hand, if carboranylporphyrins are poorly transported

by Pgp, their biodistribution should be thoroughly examined since blood–brain barrier permeability is regulated, at least in part, by Pgp.[55,56]

One mechanism of preferential uptake of porphyrins is the elevated expression of low density lipoprotein receptors (LDL receptors) in transformed cells compared with their non-malignant counterparts.[57,58] Porphyrins are accumulated in tumor cells via binding with high-affinity LDL receptors or through internalization/endocytosis upon association with low-affinity LDL receptors.[57] In non-malignant fibroblasts, the LDL receptor expression depends on cell density and was reported to be the lowest in subconfluent cells,[59] a condition maintained in our cell viability experiments. These data provide an explanation for higher sensitivity to **24** of LDL receptor positive K562 and MCF-7 cells[60,61] than of normal fibroblasts. Furthermore, drug resistant cells frequently overexpress LDL receptors, and activity of these receptors is refractory to inhibition of Pgp transport.[60] Thus, Pgp modulating agents should synergize with carboranylporphyrins in killing Pgp-positive tumor cells given that the LDL receptor mediated transport remains unaltered. This hypothesis is supported by sensitization of MCF-7Dox cells to **24** by VER.

In summary, our data demonstrate that, for tumor cells, boronated porphyrins are toxic, even as single agents; one may expect this activity to potentiate the efficacy of these compounds as photo/radiosensitizers in binary treatments. Potency of the Cu(II) salt of monocarbon carboranyl-substituted 5,10,15,20-tetraphenylporphyrin for cells with altered stress response proves the applicability of this chemical class for circumventing anticancer drug resistance.

4. Experimental

4.1. Chemistry

The solvents were purified according to standard procedures. All experiments were performed under an argon atmosphere. The reagents were from Sigma–Aldrich Fine Chemicals, St. Louis, MO, unless specified otherwise. The identities of new compounds were verified by TLC on Silufol UV 254 plates (Kavalier; Czech Republic). Column chromatography was carried out on L silica gel 40–100 μm. The electronic spectra were recorded on a Jasco UV 7800 spectrophotometer. The IR spectra were registered on Vector 22 spectrophotometer (film detection) and UR-20 spectrophotometer (detection in KBr tablets). The ^{1}H NMR spectra (400.3 MHz) were determined on a Bruker AMX-400 instrument in acetone-d_6. The mass spectra (400.3 MHz) were measured on an VISION 2000 (MALDI) spectrometer (Table 1).

4.1.1. Synthesis of compounds 6–9: General procedure. To the solution of porphyrin **1** (75 mg, 0.11 mmol) in dry CH_2Cl_2/C_5H_5N (15:12 mL), Et_3N (0.8 mL) and 20 mg DMAP were added under an argon atmosphere. The reaction mixture was stirred for 30 min, then 0.66 mmol of the respective carborane acid chloride (**2**, **3**, **4** or **5**) was added, and the stirring was continued for 3 h. After that, the reaction mixture was refluxed for an additional 1 h. The solvents were evaporated in vacuo to dryness, and the residue was purified on SiO_2 column (1×20 cm) using $CHCl_3$/CH_3OH (9:1 v/v) as an eluent, affording carboranylporphyrins **6**–**9** with 5–45% yield.

4.1.2. 5,10,15,20-Tetra{[*p*-(*o*-carboran-9-yl)methylcarbonyl]aminophenyl}porphyrin (6). Yield, 44 mg (28%). Dark red crystals. R_f 0.35. ^{1}H NMR: −2.75 (br s, 2H, porphyrin NH); 2.5 (s, 8H, CH_2); 4.13 (br s, 8H, carborane CH); 8.51–7.34 (m, 16H, Ph); 8.93 (s, 4H, amide NH); 9.34 (s, 8H, β-pyrrole).

4.1.3. 5,10,15,20-Tetra{[*p*-3-(*o*-carboran-9-yl)butylcarbonyl]aminophenyl}porphyrin (7). Yield, 70 mg (40%). Dark red crystals. R_f 0.40. ^{1}H NMR: −2.75 (br s, 2H, porphyrin NH); 0.88 (m, 16H, 9-B-CH(CH_3)); 1.40 and 1.91 (m + m, 8H, diastereotopic CH_2CH(CH_3) protons); 2.23 and 2.43 (m + m, 8H, diastereotopic $COCH_2$ protons); 4.13 (br s, 8H, carborane CH); 7.61–8.14 (m, 16H, Ph); 8.92 (s, 4H, amide NH); 9.60 (s, 8H, β-pyrrole).

4.1.4. 15,10,15,20-Tetra{[*p*-3-(*m*-carboran-9-yl)butylcarbonyl]aminophenyl}porphyrin (8). Yield, 79 mg (45%). Dark red crystals. R_f 0.45. ^{1}H NMR: −2.75 (br s, 2H, porphyrin NH); 0.91 (m, 16 H, 9-B-CH(CH_3)); 1.42 and 1.96 (m + m, 8H, diastereotopic CH_2CH(CH_3) protons); 2.26 and 2.48 (m + m, 8H, diastereotopic $COCH_2$ protons); 4.13 (br s, 8H, carborane CH); 7.19–8.56 (m, 16H, Ph); 8.94 (s, 4H, amide NH); 9.65 (s, 8H, β-pyrrole).

4.1.5. 5,10,15,20-Tetra{[*p*-(*m*-carboran-9-yl)carbonyl]aminophenyl}porphyrin (9). Yield, 7 mg (5%). Dark red crystals. R_f 0.30.

4.1.6. Synthesis of compounds 15–19: General procedure. To the solution of the respective formylporphyrin (**12**, **13** or **14**) (0.2 mmol) in THF (10 mL), the solution of lithium carborane (**10** or **11**) (0.4 mmol) was added and kept at 20 °C with stirring under argon for 2 h. The reaction mixture was then poured into water (20 mL) and extracted with CH_2Cl_2 (3×5 mL). The organic solution was washed with water, dried, and evaporated in vacuo. The residue was chromatographed on a SiO_2 column (1×20 cm) using $CHCl_3$/hexane (2:1 v/v) as an eluent, affording carboranylporphyrins **15**–**19** in 46–57% yield. Lithium carboranes **10** and **11** were prepared by treating a solution of 1-methyl-*o*-carborane or 1-isopropyl-*m*-carborane (0.4 mmol) in THF (3 mL) with BuLi (0.4 mmol, 1.3 M, hexane solution).

4.1.7. [2-(*closo*-1′-Methyl-*o*-carboranyl)hydroxymethyl-5,10,15,20-tetraphenylporphyrinato] copper (II) (15). Obtained from compound **12** (140 mg, 0.2 mmol) and compound **10** (0.4 mmol) Yield, 92 mg (53%). Dark red crystals. R_f 0.45.

4.1.8. [2-(*closo*-1′-Methyl-*o*-carboranyl)hydroxymethyl-5,10,15,20-tetraphenylporphyrinato] cobalt (II) (16). Obtained from compound **13** (140 mg, 0.2 mmol) and

compound **10** (0.4 mmol). Yield, 80 mg (46%). Dark red crystals. R_f 0.50.

4.1.9. [2-(*closo*-1′-Isopropyl-*m*-carboranyl)hydroxymethyl-5,10,15,20-tetraphenylporphyrinato] copper (II) (17). Obtained from compound **12** (140 mg, 0.2 mmol) and compound **11** (0.4 mmol). Yield, 102 mg (57%). Dark red crystals. R_f 0.55.

4.1.10. [2-(*closo*-1′-Isopropyl-*m*-carboranyl)hydroxymethyl-5,10,15,20-tetraphenylporphyrinato] cobalt (II) (18). Obtained from compound **13** (140 mg, 0.2 mmol) and compound **11** (0.4 mmol).Yield, 96 mg (54%). Dark red crystals. R_f 0.60.

4.1.11. 2-(*closo*-1′-Isopropyl-*m*-carboranyl)hydroxymethyl-5,10,15,20-tetraphenylporphyrin (19). Obtained from compound **14** (128 mg, 0.2 mmol) and compound **11** (0.4 mmol). Yield, 102 mg (57%). Dark red crystals. R_f 0.40. ^{1}H NMR: −2.65 (br s, 2H, porphyrin NH); 0.94 (d, 3H, CH_3, J = 7.0 Hz); 0.97 (d, 3H, CH_3, J = 7.0 Hz); 1.41 (m, 1H, C*H*$(CH_3)_2$, J = 7.0 Hz); 3.05 (br s, 1H, OH); 3.46 (d, 1H, C*H*-OH, J = 8.2 Hz); 7.23–8.26 (m, 20H, Ph); 8.57 (s, 1H, β-pyrrole); 8.74 (s, 6H, β-pyrrole).

4.1.12. Synthesis of compounds 20–22. General procedure. To the solution of 0.1 mmol of the respective carboranylporpohyrin (**15**, **18**, or **19**) in 5 mL THF, 0.2 mmol of Bu_4NF was added and boiled for 2 h. THF was evaporated in vacuo and hexane (10 mL) was added affording a red precipitate. The resulting solid was filtered, and then the reaction product was purified by chromatography on SiO_2 (column 1×20 cm), using the $CHCl_3/CH_3CN$ (9:1 v/v) mixture as an eluent.

4.1.13. [2-(7-Methyl-*nido*-7,8-dicarbaundecaboranylhydroxymethyl)-5,10,15,20-tetraphenylporphyrinato] copper (II) tetrabutylammonium (20). Obtained from compound **15** (88 mg, 0.1 mmol) and Bu_4NF (130 mg, 0.2 mmol). Yield, 72 mg (65%). Dark red crystals. R_f 0.35.

4.1.14. [2-(7-Isopropyl-*nido*-7,9-dicarbaundecaboranylhydroxymethyl)-5,10,15,20-tetraphenylporphyrinato] cobalt (II) tetrabutylammonium (21). Obtained from compound **17** (100 mg, 0.1 mmol) and Bu_4NF (130 mg, 0.2 mmol). Yield, 76 mg (67%). Dark red crystals. R_f 0.40.

4.1.15. 2-(7-Isopropyl-*nido*-7,9-dicarbaundecaboranylhydroxymethyl)-5,10,15,20-tetraphenylporphyrin tetrabutylammonium (22). Obtained from compound **19** (84 mg, 0.1 mmol) and Bu_4NF (130 mg, 0.2 mmol). Yield, 66 mg (62%). Dark red crystals. R_f 0.30. ^{1}H NMR: −2.6 (br s, 2H, porphyrin NH); −2.2 (br s, Δ = 0.4 ppm (carborane H_μ)); 0.95 (d, 3H, CH_3, J = 7.2 Hz); 0.98 (d, 3H, CH_3, J = 7.2 Hz); 1.42 (m, 1H, C*H*$(CH_3)_2$, J = 7.2 Hz); 3.08 (br s, 1H, OH); (d, 1H, C*H*-OH, J = 8 Hz); 7.21–8.25 (m, 20H, Ph); 8.59 (s, 1H, β-pyrrole); 8.78, (s, 6H, β-pyrrole).

4.1.16. Synthesis of compounds 24–26. General procedure. To a solution of respective formylporphyrin (**12**, **13** or **14**) (0.1 mmol) in THF (10 mL), the suspension of 1-lithium-*closo*-monocarbon carboranyl cesium (**23**) (55 mg, 0.2 mmol) in 5 mL THF was added and kept at 20 °C with stirring under argon for 1 h. The reaction mixture was then poured into the water (20 mL) and extracted with CH_2Cl_2 (3×5 mL). The organic solution was washed with water, dried, and evaporated in vacuo. The reaction product was purified by chromatography on SiO_2 (column 1×20 cm), using the $CHCl_3/CH_3CN$ (9:1 v/v) mixture as an eluent.

Compound **23** was prepared by treatment of the solution of 1-*closo*-monocarbon carboranyl cesium (0.2 mmol) in THF (5 mL) with BuLi (0.2 mmol, 1.3 M, hexane solution).

4.1.17. [2-(1′-*closo*-Monocarbon carboranylhydroxymethyl)-5,10,15,20-tetraphenylporphyrinato] copper (II) cesium (24). Obtained from compound **12** (70 mg, 0.1 mmol) and compound **23** (55 mg, 0.2 mmol). Yield, 91 mg (85%). Dark red crystals. R_f 0.40.

4.1.18. [2-(1′-*closo*-Monocarbon carboranylhydroxymethyl)-5,10,15,20-tetraphenylporphyrinato] cobalt (II) cesium (25). Obtained from compound **13** (70 mg, 0.1 mmol) and compound **23** (55 mg, 0.2 mmol). Yield, 81 mg (76%). Dark red crystals. R_f 0.45.

4.1.19. 2-(1′-*closo*-Monocarbon carboranylhydroxymethyl)-5,10,15,20-tetraphenylporphyrin cesium (26). Obtained from compound **14** (64 mg, 0.1 mmol) and compound **23** (55 mg, 0.2 mmol). Yield, 63 mg (70%). Dark red crystals. R_f 0.35. ^{1}H NMR: −2.74 (br s, 2 H, porphyrin NH); 3.01 (d, 1H, OH, J = 8.0 Hz); 3.75 (d, 1H, C*H*-OH, J = 8.0 Hz); 7.9–8.3 (m, 20H, Ph); 8.64 (s, 1H, β-pyrrole); 8.74 (s, 6H, β-pyrrole).

4.2. Biology

4.2.1. Cell lines and drugs. All cell lines used in this study were of human origin. The K562 leukemia cell line (American Type Culture Collection, ATCC, Manassas, VA) and its subline K562i/S9 (gift of I. Roninson, Chicago, USA) were propagated in RPMI-1640 supplemented with 5% fetal bovine serum (BioWhittaker, Belgium), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C, 5% CO_2 in humidified atmosphere. The K562i/S9 subline expresses Pgp after infection of K562 cells with a retrovirus carrying full length *MDR*1/Pgp cDNA, followed by flow cytometry based sorting of Pgp-positive cells.[37,38] The K562i/S9 cells have not been selected with any drug. The MCF-7 breast carcinoma cell line (ATCC) and its variant MCF-7Dox selected for growth in the presence of DOX (gift of T. Ignatova, Chicago, USA), CaOv ovarian cancer cell line, A2780 ovarian cancer cell line (both from ATCC), and its subline A2780DDP selected for resistance to cisplatin (gift of A. Burger, Freiburg, Germany) were cultured in Dulbecco's modified Eagle's medium supplemented as above. The LNCaP prostate cancer cell line (ATCC), FEMX melanoma cell line and its variant FEMX/D selected for resistance to growth inhibitory effect of dexamethasone (gift of M. Krasilnikov, Moscow, Russia), and HCT116 colon carcinoma cell line ($p53^{+/+}$) and its variant with deletion of both alleles of p53 (HCT116p53KO) (provided by B. Kopnin, Moscow, Russia) were grown in RPMI-1640

with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The LNCaP-abl subline selected for long-term androgen ablation (gift of H. Klocker, Innsbruck, Austria) was propagated in the same medium supplemented with charcoal treated fetal calf serum. All cell lines were routinely tested and found free from *Mycoplasma*. The cultures in logarithmic phase of growth were used in all experiments. The skin fibroblasts were early (2–3) passage cultures from thinly minced pieces of intact skin dissected at surgery. Informed patient's consent was obtained. Drugs (including novel carboranylporphyrins) were dissolved as 1000× stock solutions in water or dimethylsulfoxide (DMSO) immediately before experiments. All porphyrin-containing compounds were kept away from light. The experiments with porphyrins were performed in the dark.

4.2.2. Cell viability assay. The toxicity of novel compounds was determined in a MTT test.[62] Cells (3×10^3 in 100 μL of culture medium) were plated into a 96-well plate (Becton Dickinson, Franklin Lakes, NJ) and treated with vehicle control (0.1% DMSO), conventional anticancer drugs or novel carboranylporphyrins (each concentration in triplicate) for 72 h. The carboranylporphyrins were added to cell cultures in the dark. In the experiments with inhibition of Pgp-mediated transport, VER (20 μM) was added to the wells simultaneously with **24**. The final concentration of DMSO in the medium did not exceed 0.1%. This dose of DMSO caused no discernible toxicity, growth arrest, or any morphological changes in cells within the time frame of experiments (not shown). After the completion of drug exposure, 100 μg MTT in 20 μL of aqueous solution was added into each well for an additional 2 h. Formazan was dissolved in acidified DMSO, and the absorbance at $\lambda = 540$ nm was measured on a Flow Multiscan plate reader (LKB, Sweden). Cell survival was calculated as the ratio of OD_{540} in wells with the respective drug concentrations to OD_{540} of wells containing vehicle control (100%).

4.2.3. Immunoblot analysis of Pgp. Cells were lysed in buffer containing 30 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], 10 mM NaCl, 5 mM $MgCl_2$, 25 mM NaF, 1 mM tetrasodium ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′*N*′-tetraacetate, 1% Triton X-100, and 10% glycerol, 2 mM Na orthovanadate, 2 mM phenylmethylsulfonyl fluoride, aprotinin, and leupeptin (10 μg/mL each) for 30 min on ice. The lysates were centrifuged (10,000*g*, 7 min), and supernatants were used for further procedure. Thirty microgram of total protein (determined by a bicincholinic method; Pierce, Rockford, IL) was resolved by electrophoresis in a 7% polyacrylamide/0.1% sodium dodecyl sulfate gel (SDS–PAGE) and electroblotted on a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked with 5% non-fat dried milk in buffer containing 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 followed by incubation with mouse anti-Pgp antibody (1:1000) and horseradish peroxidase-conjugated anti-mouse IgG (1:2500). Proteins were visualized using Enhanced Chemoluminescence System (Amersham Pharmacia Biotech., UK). After stripping, the membranes were incubated with anti-β-actin antibody (internal standard) (1:5000) and processed as described above.

4.2.4. Analysis of Pgp-mediated transport. The MCF-7 and MCF-7Dox cells grown in 35 mm petri dishes (5×10^5 cells in 2 mL of culture medium) were left unloaded (control) or treated with 300 nM rhodamine 123 for 1 h at 37 °C. A parallel portion of MCF-7Dox cells was treated with rhodamine 123 and 20 μM VER. Then, the monolayers were washed with ice-cold phosphate-buffered saline, pH 7.2 (PBS), detached from plastic, resuspended in PBS, and immediately analyzed by flow cytometry on FL1 (FACSCalibur, Becton Dickinson, San Jose, CA). Five thousand events were collected for each treatment. Data were analyzed using CellQuest™ software.

4.2.5. Morphological signs of cytotoxicity of compound 24. The MCF-7Dox cells were plated on glass coverslips, allowed to reach ~50% confluence, and then treated with 0.05% DMSO (control) or 4 μM **24** + 20 μM VER for 5–24 h. Cells were washed with PBS, fixed in methanol, stained with azur and eosin, and visualized in Axiolab microscope (Carl Zeiss, Germany).

Statistical analysis was performed using Student's *t* test.

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Note added in proof

When this article was under review, it was shown in the E. DeClercq laboratory (Rega Institute of Medical Research, University of Leuven, Belgium) that compound **24** is toxic for CEM, Molt-4 and L1210 leukemia cell lines at low micromolar concentrations. These data re-iterate that crboranylporphyrins and their metal complexes can be candidate anticancer drugs along with their use in binary treatment strategies.

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