Synthesis of C_{60} Fullerene—Quadricyclane Hybrid Compound and Its Preliminary In Vitro Antitumor Activity in Combination with Cisplatin

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ABSTRACT: This paper describes the authors’ preliminary studies directed toward the possibility of the practical implementation of the idea to design efficient antitumor drugs based on hybrid molecules composed of fullerene C_{60} and quadricyclanes. The essence of the proposed idea is that these hybrid molecules are able to cleave DNA owing to the fullerene moiety they contain and to simultaneously thermally affect tumor cells via cleavage of the carbon–carbon bond in quadricyclanes under the action of Pt and Pd ions. As a result, testing of the cytotoxic activity in vitro for a number of fullerene C_{60} hybrids with the norbornadiene or quadricyclane moieties against the human T-lymphoblastic leukemia cells (Jurkat cells) in combination of the known cisplatin drug, which was taken as the source of Pt ions, showed a statistically reliable dose-dependent increase in the number of dead cells in each group, which were formed according to the amount of cisplatin added, in comparison with the control, that is, cells treated with cisplatin or quadricyclane fullerene derivatives alone. Indeed, the difference between the percentages of viable cells after treatment with either cisplatin alone or cisplatin in combination with methanofullerene $\Delta$ ranged from $\sim$10% (for Pt (0.015 mkM), $\Delta$ (0.015 mkM)) to $\sim$55% (for Pt (0.03 mkM), $\Delta$ (0.045 mkM)).

INTRODUCTION

The unique properties of fullerenes and their derivatives have been attracting close attention of researchers in relation to the development of efficient antioxidants,\textsuperscript{1} solar energy converters,\textsuperscript{2} artificial diamonds,\textsuperscript{3,4} materials for electronic,\textsuperscript{5} semiconductor equipment,\textsuperscript{6} engine oil additives,\textsuperscript{7} and advanced pharmaceutical agents.\textsuperscript{8} Of particular interest and practical value are biologically active fullerene derivatives with potential for the development of new-generation drugs needed for the treatment of socially significant diseases.

The biological activity of fullerenes stems is attributed to number of their properties such as lipophilicity (responsible for membranotropic behavior), electron deficiency (determines the reactivity toward free radicals), and the ability of fullerenes in the excited state to transfer energy to a dioxygen molecule, which is thus converted to singlet oxygen.\textsuperscript{9} Research into the antitumor activity of fullerenes occupies a special place in the whole range of their possible applications.

Testing in mice has demonstrated the ability of fullerene C_{60} to suppress the growth of various histological types of tumors.\textsuperscript{10,11} In 2010, Jiao and co-workers\textsuperscript{12} found that poly hydroxyfullerene C_{60}(OH), activates the peritoneal macrophages and inhibits the tumor growth in the EMT-6 metastatic breast cancer model. In turn, the quaternary ammonium salt of simple pyrrolidinofullerene exhibited high cytotoxicity toward promyelocyte leukemia cells.\textsuperscript{13,14} More comprehensive descriptions of antitumor activity assays for fullerenes and their derivatives were reported by Bolskar and Orlova and co-workers.\textsuperscript{15,16} Particular attention of researchers is on the use of fullerenes for the targeted delivery of various substances\textsuperscript{17} and enhancement, in this way, of the antitumor properties\textsuperscript{18–20} of well-known drugs. The use of fullerenes as targeted drug delivery vehicles is a relatively new; however, it is a vigorously developing trend in fullerene chemistry and applications, which requires both new ideas and long-term systematic research.

Recently,\textsuperscript{21} we have synthesized cyclopropane fullerene C_{60} derivatives containing norbornadiene and quadricyclane moieties. It was found that in the presence of a catalytic amount of cisplatin, quadricyclane-containing methanofullerenes are quantitatively isomerized to the corresponding norbornadienes with heat generation. In view of the discovered carbon–carbon bond cleavage in the quadricyclane moiety of the hybrid molecule in the presence of catalytic amount of Pt ions with the generation of about 110 kJ/mol\textsuperscript{22} of heat, we
proposed the idea that new hybrid molecules would be accumulated more efficiently in tumor cells than in normal cells because of more intense tumor cell metabolism. On subsequent introduction of cisplatin in a substantially lower amount than used in medicine, the hybrid molecules will be cleaved and hence exert simultaneously both chemotherapeutic and thermal effects on cancer cells, which will induce, as we hope, efficient cell death.

**RESULTS AND DISCUSSION**

To test our hypothesis, here we report the synthesis of the hybrid molecule comprising fullerene C₆₀ and qudricyclic by the Bingel–Hirsch reaction. In view of the high hydrophobicity of fullerens and fullerene derivatives and low stability of the qudricyclic moiety located in the close vicinity to the fullerene core, malonic acid ester 3 was synthesized as the precursor of the α-halo carbamions. Scheme 1 depicts the total synthetic route to the hybrid molecule, which includes the subsequent introduction of hydrophilic monoacylatedtriethylene glycol to norbornadiene malonic acid monoester 1 by the carbodiimide method and UV irradiation of the norbornadiene malonic ester 2 to give qudricyclic ester 3. In the final stage, nucleophilic addition of α-halo carbamions generated in situ by the reaction of qudricyclic ester 3 with CBr₄ in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) resulted in the synthesis of the target hybrid molecule 5 in ∼68% yield. To identify the effect of the qudricyclic moiety on the antitumor activity of hybrid molecule 5, methanofullerene 4 with the norbornadiene addend was synthesized as the reference compound.

The structures and compositions of methanofullerenes 4 and 5 were reliably established using modern physicochemical techniques (nuclear magnetic resonance (NMR), UV, and matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF)/TOF mass spectrometry).

The ¹³C NMR spectrum of methanofullerene 4 shows 14 nonequivalent signals corresponding to the type of symmetry of the fullerene molecule, which has two local planes of symmetry, one coinciding with the cyclopropane ring plane and the other passing orthogonal to this plane through the common bridging carbon atom. The norbornadiene moiety is manifested as characteristic ¹H NMR doublets at 2.04 and 2.07 ppm for the geminal protons that are linked, according to the HMBC experiment, to the bridging carbon atom with δc of 70.55 ppm, and as multiplets with δH 6.92 and 7.01 ppm for the double-bond hydrogen atoms of the polycycle. The ¹H NMR spectrum of methanofullerene 5, unlike that of norbornadiene isomer 4, exhibits a set of high-field signals at about 1.5–2.7 ppm, corresponding to the cage hydrogen atoms, which is in full agreement with the published data for the qudricyclic adducts.

In the next stage of our research, we have investigated the in vitro antitumor activity of the norbornadiene and qudricyclic derivatives of fullerene C₆₀ 4 and 5 on the human T-lymphoblastic leukemia cells (Jurkat cells).

According to the published data, fullerene C₆₀ enhances the cytotoxicity of cisplatin against the HL-60/adr and HL-60/vinc tumor cells, including cisplatin-resistant sublines; therefore, we initially studied the cytotoxicity of an aqueous solution of the polyvinylpyrrolidone complex of fullerene C₆₀ used in combination with a cisplatin solution in dimethylformamide and the effects of methanofullerenes 4 and 5 with qudricyclic and norbornadiene substituents on Jurkat cells.

Treatment of the cells with aqueous solutions of the poly(vinylpyrrolidone) complex or methanofullerene 5 in different concentrations (0.015, 0.03, and 0.045 mkM) does not affect significantly the cell viability even after 72 h (Figure S11).

Simultaneously, it was shown that the combined addition of cisplatin and a solution of C₆₀ does not induce a significant change in the Jurkat cell viability as compared with the cells treated with cisplatin alone (Figure S12).

Having obtained the above control parameters of the cytotoxic effect of the initial compounds and cisplatin on the viability of Jurkat cells, in the next stage, we made efforts to experimentally verify the hypothesis, put forward previously, about the utilization of the exothermic conversion of qudricyclic to norborna-2,5-diene under the action of catalytic amounts of platinum compounds for the potential use in the therapy of cancer.

Initially, the Jurkat cells were treated simultaneously with solutions of methanofullerene 5 and cisplatin (Pt) in various concentrations (Pt, 0, 0.015, 0.03, and 0.045 mM; Pt, 0, 0.015, 0.03, 0.06 mM); after incubation for 24 h, the cells were stained with 7-aminoactinomycin D (AAD), and the ratio of dead and viable cells was determined by flow cytometry and is depicted as a histogram in Figure S13. The results indicated the absence of any effect of the joint treatment of tumor cells with cisplatin and methanofullerene 5. The percentages of viable cells in each group of cisplatin concentrations (0.015, 0.03, and 0.06 mM) at various concentrations of compound 5...
(0, 0.015, 0.03, 0.045 mM) differed by 2–5%, which is within the experimental error.

Considering the high rate of quadricyclane ring opening induced by platinum and palladium compounds,24,25 we assumed that in the case of simultaneous addition of cisplatin and methanofullerene 5, the catalytic conversion of quadricyclane to norbornadiene occurred outside the cell, which accounts for the failure. Therefore, in the subsequent studies, we first incubated the cells with cycloadduct 5 in various concentrations for 24 h, then a cisplatin solution (0.015, 0.03, or 0.06 mM) was added, and this was followed by one more incubation for 24 h. After the treatment of the flow cytometry results, as shown below in Figure 1, a reliable dose-dependent increase in the number of dead cells was detected in each group (formed according to the amount of cisplatin added) in comparison with the control, that is, the cells treated with cisplatin alone (Figure 1).

Indeed, the difference between the percentages of viable cells after treatment with either cisplatin alone or cisplatin in combination with methanofullerene 5 ranged from ~10% (for Pt (0.015 mM), 5 (0.015 mM)) to ~55% (for Pt (0.03 mM), 5 (0.045 mM)). It is known that quadricyclanes (without fullerene C60) kill ~64% of Jurkat cancer cells at a concentration of 0.3 mM used.26

It is noteworthy that conducting this experiment with methanofullerene 4 containing a norbornadiene moiety did not reveal a significant increase in the percentage of dead cells in comparison with the control.

We have also tested our hypothesis on normal fibroblasts (Figure 2). When compound 5 was added separately with the subsequent addition of cisplatin after 24 h of incubation, the changes in the culture of normal fibroblasts were similar to those of Jurkat cells treated and incubated in a similar manner. Meanwhile, statistical data processing showed a reliable increase in the number of viable cells in the fibroblast culture at similar concentrations of compound 5 and cisplatin as compared to the Jurkat cells (the concentration of compound 5 was 0.015, 0.03, and 0.045 mM, respectively) (p ≤ 0.005).

The hypothesis about the cell thermal necrosis due to the heat released during the intracellular isomerization of the quadricyclane fragment of the fullerene derivative 5 under the action of cisplatin is also confirmed by the results of the investigations into the induction of apoptosis using flow cytometry (Figure S14).

Thus, to study the induction of apoptosis, before starting the flow cytometry experiment, the chosen cell line was treated by fluorescent dyes (annexin V and propidium iodide (PI)). This allowed to select four different cell populations, as a percentage, when registering the process of apoptosis: living cells (Q7-3, annexin V−/PI−), early cell apoptosis (Q7-4, annexin V+/PI−), late apoptosis (Q7-2, annexin V+/PI+) and necrosis (Q7-1, annexin V−/PI+). As shown in Figure 3, treatment of Jurkat cells with quadricyclane 5 (0.045 mM) followed by incubation for 24 h has practically no effect on the cell population as compared to the control in Figure 3A. When cells were treated with cisplatin (0.06 mM) and exposure time was 24 h, only a population of living cells (32%), as well as cells in early (12%) and late (56%) apoptosis, were observed (Figure 3C). In turn, a cytometric picture underwent significant changes, when adding cisplatin (0.015 mM) to cells preincubated for 24 h with quadricyclane 5 (0.045 mM), with exposure time of 2 h. The cytometry data have shown populations of only living and necrotic cells (Figure 3D). This is in good agreement with our idea of cell thermal necrosis as a result of the intracellular isomerization of the quadricyclane fragment of fullerene derivative 5 under the action of cisplatin due to the heat release.

### CONCLUSIONS

We have synthesized hybrid molecules based on fullerene C60 and norbornadiene or quadricyclane using the Bingel–Hirsch reaction. In a study of the cytotoxic effect of hybrid molecules together with cisplatin on the T-lymphoblastic leukemia cells (Jurkat cells), we have demonstrated for the first time that the water-soluble poly(vinylpyrrolidone) complex of methanofullerene containing the quadricyclane addend induces a statistically reliable increase in the number of dead cells in...
each group, formed according to the amount of cisplatin added, in comparison with the control.

**EXPERIMENTAL SECTION**

**General Information.** All reactions were performed under an argon atmosphere and in anhydrous solvent. The solvents and reagents were dried or refined according to the literature procedures. Commercially available [60]fullerene (99.5% pure, Sigma-Aldrich) and cisplatin (ABCR) were used. The reaction products were analyzed on a high-performance liquid chromatography (HPLC) chromatograph Shimadzu SPD-20A (Japan) equipped with the UV detector at 313 or 340 nm. The mixtures were separated on a preparative column Cosmosil Buckyprep Waters (250 × 10 mm²) at ~20 °C. Toluene was used as eluent, and the flow rate was 3.0 mL/min. The ¹H and ¹³C NMR spectra were recorded on a Bruker Avance-500 spectrometer at 500.17 and 125.78 MHz, respectively. A mixture of CDCl₃ and CS₂ (1:5) was used as a solvent. The chemical shifts are reported as δ values in parts per million relative to the internal standard Me₄Si. The coupling constants (J) are reported in Hertz. The mass spectra were obtained on an UltraFlex III TOF/TOF (Bruker Daltonik GmbH, Germany) operating in linear (TOF) and reflection (TOF/TOF) positive and negative ion modes. S₈ and DCTB (trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenyliden]malononitrile) were used as the matrix. For the application on a metal target, toluene solutions of the samples were used. Hamamatsu Lightning cure LC-8 150 W was used for UV irradiation of norbornadienes. Elemental analyses were measured on a 1106 Carlo Erba apparatus.

**Procedure for the Synthesis of Compound 2.** A 50 mL glass reactor was charged with ester 1 (3.79 mmol) in dry dichloromethane (30 mL), monoacylatedtriethylene glycol (4.17 mmol), DCC (3.79 mmol), and 4-dimethylaminopyridine (0.379 mmol) at room temperature. After 2 h, the reaction mixture was passed through a column with small amount of silica gel and concentrated in vacuo. The product was purified by column chromatography, eluent hexane/EtOAc 1:3. Yields 54%, light yellow oil. UV (i-PrOH), λ_max nm: 217, 269. ¹H NMR (500 MHz, CDCl₃, δ): 2.06 (s, 3H, CH₃), 2.07 and 2.25 (both d, J = 6.5 Hz, 2H, CH₂), 3.38 (s, 2H, CH₂), 3.69 (m, 8H, 4CH₂), 3.84 and 4.05 (both broad s, 2H, CH₂), 4.20 (m, 2H, CH₂), 4.29 (broad s, 6H, 3CH₂), 6.91 and 6.98 (both t, J = 3.5 Hz, 2H, 2CH), 7.30 (m, 3H, 3CH), 7.49
Preparative Photoisomerization (General Procedure). The norbornadiene 2 was dissolved in acetone and irradiated with a 150 W UV Lamp for 60 min. The photoisomerization was confirmed by 1H and 13C NMR spectroscopy. The product was not isolated.

Yield 100%, colorless oil. 1H NMR (500 MHz, CDCl3, δ): 1.70 (d, J = 3.5 Hz, 1H, CH), 2.06 (s, 3H, CH3), 2.10 and 2.38 (both d, J = 12.0 Hz, 2H, CH2), 2.24 (broad s, 1H, CH), 2.48 (d, J = 3.5 Hz, 1H, CH), 2.60 (broad s, 1H, CH), 3.36 (s, 2H, CH2), 3.69 (m, 6H, 3CH2), 4.12 (broad s, 4H, 2CH2). 13C NMR (125 MHz, CDCl3, δ): 20.7, 29.8, 31.4, 31.7, 32.1, 32.7, 37.5, 41.1, 61.1, 63.1, 63.5, 64.5, 68.8, 69.1, 70.50, 70.54, 126.2, 127.7, 128.6, 135.4, 138.9, 139.1, 140.7, 140.9, 141.0, 141.8, 141.9, 142.2, 143.0, 143.7, 143.8, 144.6, 144.9, 145.0, 145.29, 163.4, 164.7, 168.4, 171.0. HRMS (MALDI TOF), m/z 1234.1839 [M]+. Yield 94 mg (55%), brown powder.

General Procedure for the Synthesis of Norbornadiene and Quadracyclic Containing Methanofullerenes 4 and 5. A 50 mL glass reactor was charged with C60 (100 mg, 0.139 mmol) in o-xylene (12 mL), corresponding to malonic ester (0.2085 mmol), CBr4 (69 mg, 0.2085 mmol), and DBU (0.031 mL, 0.2085 mmol) at room temperature. The resulting reaction mixture was stirred for 1 h and quenched with water. The layers were separated and the organic layer was passed through a column with small amount of C18 silica gel. The reaction products and the starting fullerene C60 were separated by the semipreparative HPLC, using toluene as an eluent. The samples were washed with hexane and Et2O before NMR analysis.

Methanofullerene (4). Yield 94 mg (55%), brown powder. UV (CHCl3, λmax nm: 260, 330, 427. 1H NMR (500 MHz, CDCl3, δ): 2.06 and 2.24 (both d, J = 4.5 Hz, 2H, CH2), 2.09 (s, 3H, CH3), 3.57–3.67 (m, 12H, 6CH2), 3.88 (m, 1H, CH), 4.28 (m, 1H, CH), 4.49 (m, 2H, CH2), 4.72 (m, 2H, CH2), 6.92 (t, J = 4.5 Hz, 1H, CH), 7.01 (t, J = 4.5 Hz, 1H, CH), 7.35 (m, 5H), 7.40 (m, 2H, 2CH), 7.58 (d, J = 8.0 Hz, 2H, 2CH). 13C NMR (125 MHz, CDCl3, δ): 20.7, 20.9, 29.8, 31.4, 31.7, 32.1, 32.7, 37.5, 41.1, 61.1, 63.1, 63.5, 64.5, 68.8, 69.1, 70.50, 70.54, 126.2, 127.6, 128.7, 136.8, 166.1, 166.3, 171.0, 171.6.

Methanofullerene (5). Yield 89 mg (52%), brown powder. UV (CHCl3, λmax nm: 260, 327, 427. 1H NMR (500 MHz, CDCl3, δ): 1.72 (m, 1H, CH), 2.03 (s, 3H, CH3), 2.13 (m, 1H, CH), 2.25 (m, 1H, CH), 2.36 (m, 1H, CH), 2.51 (m, 1H, CH), 2.61 (m, 1H, CH), 4.3–4.7 (m, 16H, 8CH2), 7.21 (m, 2H, 2CH), 7.40 (m, 2H, 2CH), 7.57 (m, 1H, CH). 13C NMR (125 MHz, CDCl3, δ): 21.0, 29.7, 30.1, 31.3, 31.8, 32.2, 32.9, 37.7, 51.8, 63.5, 66.2, 68.8, 70.6, 70.5, 71.3, 127.7, 127.9, 128.9, 135.4, 138.2, 139.0, 140.7, 140.9, 141.0, 141.8, 142.2, 143.0, 143.7, 143.8, 144.6, 144.9, 145.0, 145.29, 163.4, 164.7, 168.4, 171.0. HRMS (MALDI TOF), m/z 1234.1839 [M]+. Yield 89 mg (52%), brown powder.

Biological Assays. Cell Culturing. Jurkat cells were purchased from Russian Cell Culture Collection (Institute of Cytology of the Russian Academy of Sciences) and cultured according to standard mammalian tissue culture protocols and sterile technique. All cell lines used in the study were tested and shown to be free of mycoplasma and viral contamination. Jurkat cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Gibco) supplemented with 4 mM glutamine, 10% fetal bovine serum (FBS, Sigma), and 100 units/mL penicillin-streptomycin (Sigma). Cells were grown in an atmosphere of 5% CO2 at 37 °C. The cells were subcultured at 2–3 days intervals. The cells were then seeded in 24-well plates at 5 × 104 cells per well and incubated overnight. Jurkat cells were subcultured at 2 day intervals with a seeding density of 1 × 105 cells per 24-well plates in RPMI with 10% FBS.

Cytotoxicity Assay. Viability (live/dead) assessment was performed by staining cells with 7-AAD (7-aminoactinomycin D) (Biolegend). Cells after treatment with compounds at various concentrations and incubated in an atmosphere of 5% CO2 at 37 °C during 24 h (cisplatin added simultaneously) or 24 + 24 h (cisplatin added after 24 h) were harvested, washed 1–2 times with phosphate-buffered saline (PBS), and centrifuged at 400g for 5 min. Cell pellets were resuspended in 200 µL of flow cytometry staining buffer (PBS without Ca2+ and Mg2+, 2.5% FBS) and stained with 5 µL of 7-AAD (7-aminoactinomycin) for 15 min at room temperature in the dark. Samples were acquired on NovoCyteTM 2000 FlowCytometry System (ACEA) equipped with 488 nm argon laser. Detection of 7-AAD emission was collected through a 675/30 nm filter in FL4 channel.

ASSOCIATED CONTENT

S Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01982.

Copies of MALDI, 1H NMR, and 13C NMR spectra of final products (PDF)

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Notes

The authors declare no competing financial interest.

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