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# 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 communication describes preliminary studies of the authors directed towards the possibility of 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 simultaneously to thermally affect tumor cells via cleavage of the carbon-carbon bond in quadricyclanes under the action of Pd and Pt 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.


## Keywords

Fullerene; quadricyclane; hybrid molecule; cisplatine; antitumor drugs


## Introduction

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

The biological activity of fullerenes stems from a number of their properties such as lipophilicity (responsible for membranotropic behavior), electron deficiency (determining the reactivity towards 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.[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.[10,11] In 2010, Jiao and co-workers[12] found that polyhydroxyfullerene $C_{60}(OH)_x$ 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

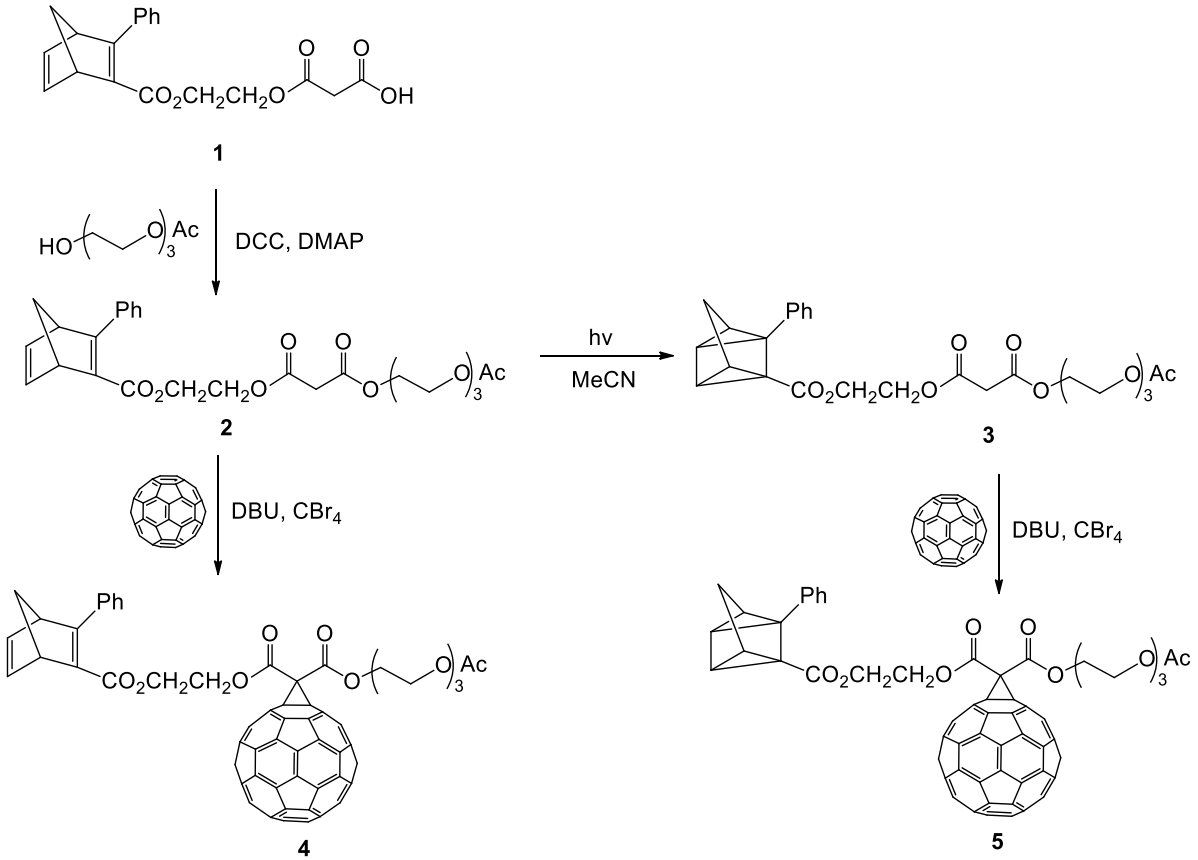
towards promyelocyte leukemia cells.[13,14] More comprehensive descriptions of antitumor activity assays for fullerenes and their derivatives were reported by Bolskar (2013) and Orlova and co-workers (2016).[15,16] Particular attention of researchers is attracted by the use of fullerenes for targeted delivery of various substances[17] and enhancement, in this way, of the antitumor properties[18-20] of well-known drugs. The use of fullerenes as targeted drug delivery vehicles is a relatively new, although vigorously developing trend in fullerene chemistry and applications, which requires both new ideas and long-term systematic research.

Recently,[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 with heat evolution to the corresponding norbornadienes. 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 evolution of about 110 kJ/mol[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 the chemotherapeutic and thermal effect on cancer cells, which will induce, as we hope, efficient cell death.

# Results and Discussion

In order to test our hypothesis, here we report the synthesis of the hybrid molecule comprising fullerene $C_{60}$ and quadricyclane by the Bingel–Hirsch reaction. In view of the high hydrophobicity of fullerenes and fullerene derivatives and low stability of the

quadricyclane moieties located in the close vicinity to the fullerene core,[21] malonic acid ester **3** was synthesized as the precursor of the α-halo carbanion. Scheme 1 depicts the total synthetic route to the hybrid molecule, which includes the subsequent introduction of hydrophilic monoacylated triethylene glycol to norbornadiene malonic acid monoester **1**[21] by the carbodiimide method, UV irradiation of the norbornadiene malonic ester **2** to give quadricyclane ester **3**. At the final stage, nucleophilic addition of α-halo carbanions generated *in situ* by the reaction of quadricyclane ester **3** with $CBr_4$ 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. In order to identify the effect of the quadricyclane moiety on the antitumor activity of hybrid molecule **5**, methanofullerene **4** with the norbornadiene addend was synthesized as the reference compound.




**Scheme 1:** Total synthetic route to methanofullerenes **4** and **5** containing norbornadiene and quadricyclane moieties.

The structures and compositions of methanofullerenes **4** and **5** were reliably established using modern physicochemical techniques (NMR, UV spectroscopy, MALDI TOF/TOF mass spectrometry).

The $^{13}C$ NMR spectrum of methanofullerene **4** shows 14 non-equivalent 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 $^{1}H$ 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 $\delta_C$ of 70.55 ppm, and as multiplets with $\delta_H$ 6.92 and 7.01 ppm for the double bond hydrogen atoms of the polycycle.

The $^{1}H$ 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 quadricyclane adducts.[23]

In the next stage of our research, we have investigated the *in vitro* antitumor activity of the norbornadiene and quadricyclane derivatives of fullerene $C_{60}$ **4** and **5** on the human T-lymphoblastic leukemia cells (Jurkat cells).

According to published data,[20] fullerene $C_{60}$ 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 $C_{60}$ used in combination with a cisplatin solution in DMF and the effects of methanofullerenes **4** and **5** with quadricyclane and norbornadiene substituents on Jurkat cells.

Treatment of the cells with aqueous solutions of the polyvinylpyrrolidone complex or methanofullerene **5** in different concentrations (0.015, 0.03, and 0.045 μM) does not affect significantly the cell viability even after 72 h (Fig. 1 in ESI).

Simultaneously it was shown that the combined addition of cisplatin and a solution of $C_{60}$ does not induce a significant change in the Jurkat cell viability as compared with the cells treated with cisplatin alone (Fig. 2 in ESI).

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 utilization of the exothermic conversion of quadricyclane 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 (**5**, 0, 0.015, 0.03, and 0.045 μM; **Pt**, 0, 0.015, 0.03, 0.06 μM); after incubation for 24 h, the cells were stained with 7-AAD, and the ratio of dead and viable cells was determined by flow cytometry and is depicted as a histogram in Fig. 3 in ESI. 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 μM) at various concentrations of compound **5** (0, 0.015, 0.03, 0.045 μM) 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 hours, then a cisplatin solution (0.015, 0.03, or 0.06 μM) was added, and this was followed by one more incubation for 24 h. After treatment of the flow cytometry results, as shown below in Fig. 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 (Fig. 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 μM), **5** (0.015 μM)) to ~55% (for Pt (0.03 μM), **5** (0.045 μM)).

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 (Fig. 2).

When compound **5** was added separately with the subsequent (followed by) addition of cisplatin after 24 hours of incubation, the changes in 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 μM, respectively) ($p \leq 0.005$).

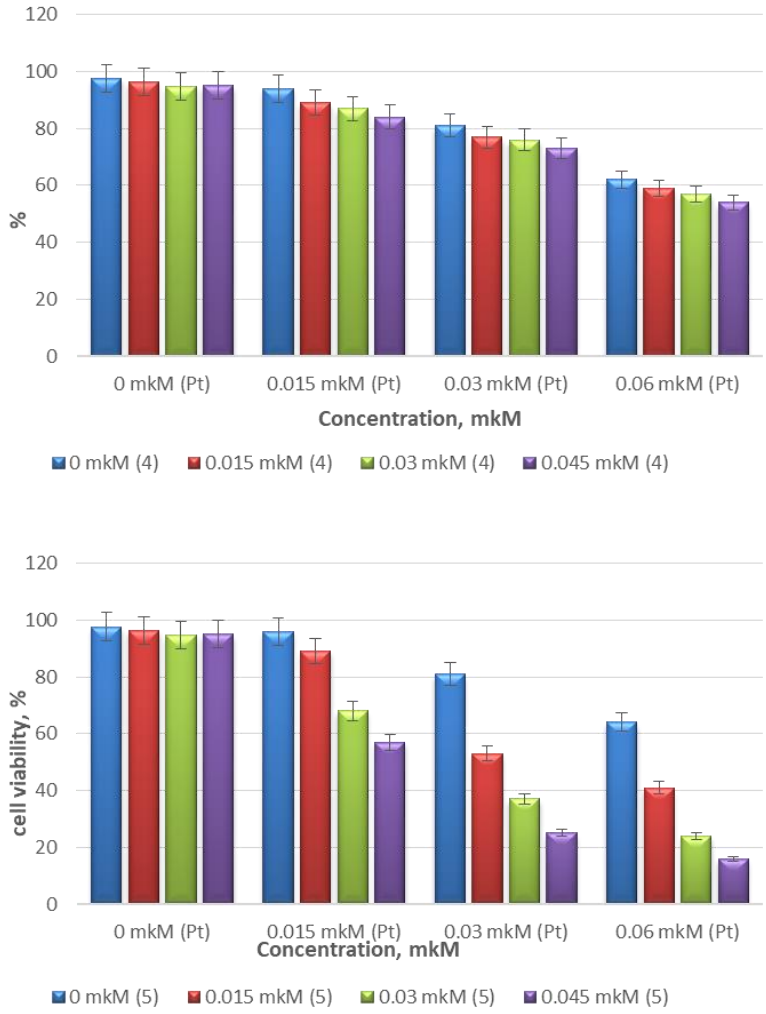



**Figure 1:** Joint effect of concentrations of methanofullerene **4** (on top) or **5** (below) and cisplatin added after 24 h on the viability of Jurkat cells (the histogram shows the percentage of viable cells).

The hypothesis about 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 induction of apoptosis using flow cytometry (Figure 4 in ESI).

Thus, to study the induction of apoptosis, before starting the flow cytometry experiment, the chosen cell line was treated by fluorescent dyes (annexine V and 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 4B (in ESI), treatment of Jurkat cells with quadricyclane **5** (0.045 μM) followed by incubation for 24 hours has practically no effect on the cell population as compared to the control in Figure 4A (in ESI). When cells were treated with cisplatin (0.06 μM) and exposure time was 24 hours, only a population of living cells (32%), as well as cells in early (12%) and late (56%) apoptosis, were observed (Fig. 4C in ESI). In turn, a cytometric picture underwent significant changes, when adding cisplatin (0.015 μM) to cells preincubated for 24 hours with quadricyclane **5** (0.045 μM), with exposure time of 2 hours. The cytometry data have shown populations of only living and necrotic cells (Fig. 4D in ESI). 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.

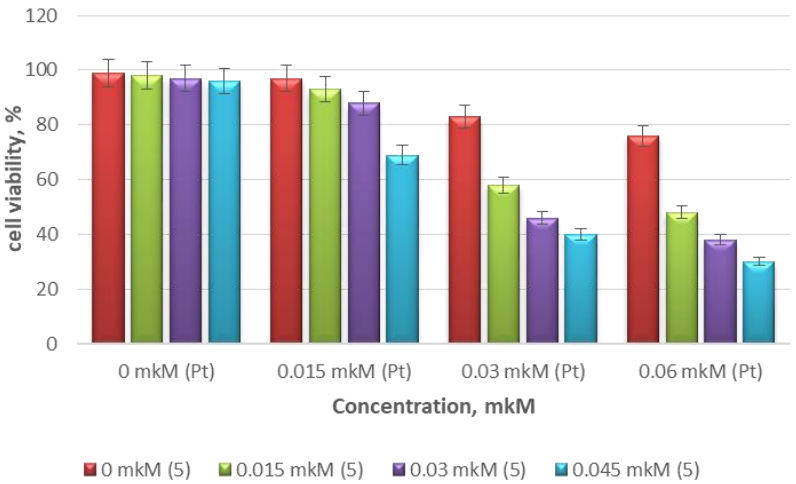



**Figure 2:** Joint effect of concentrations of **5** and cisplatin added after 24 h on the viability of normal fibroblasts (the histogram shows the percentage of viable cells).

# Conclusion

Thus, we have synthesized hybrid molecules based on fullerene $C_{60}$ 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 polyvinylpyrrolidone 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.

# Supporting Information

Supporting information

Supporting Information File 1:

File Name:

File Format:

Title:

# Acknowledgements

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for the Pharmaceutical Industry at the Institute of Petrochemistry and Catalysis of RAS.

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