УДК 616-006.6-085.27.099
https://doi.org/10.20538/1682-0363-2022-2-60-66

Evaluation of the cytotoxic activity and toxicity of a tropolone derivative with a potential antitumor effect

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ABSTRACT

The aim. To study the toxicity of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone *in vitro* and *in vivo*.

Materials and methods. 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone was synthesized using a method for expanding the *o*-quinone cycle during the reaction between 5-nitro-2,6,8-trimethyl-4-chloroquinoline and 3,4,5,6-tetrachloro-1,2-benzoquinone while boiled in dioxane. An *in vitro* experiment was carried out in the human A549 cell line. Cell viability was assessed using the MTT colorimetric assay by reducing the optical density of the experimental samples compared with the control ones. Acute toxicity was studied on 20 BALB/c Nude male mice. The test compound was administered once orally as a suspension in 1% starch gel at three doses: 0.0055 (group 1), 0.055 (group 2) and 0.55 mg / g (group 3). The control group (group 4) received a placebo.

Results. We synthesized a new compound, 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone. Its structure was established by ^{1}H nuclear magnetic resonance (NMR), infrared (IR) spectroscopy, and mass spectrometry. The yield was 19.8 g (52%), the melting point was 205–207 ºC, bright yellow crystals (benzene) were observed. The half-maximal inhibitory concentration (IC_{50}) of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone was 0.21 ± 0.01 μM, which was significantly lower ($p < 0.05$) than the IC_{50} of cisplatin (3.84 ± 0.23). Following the *in vivo* experiment, no toxic effect of tropolone was detected when administered once at a dose of 0.0055, 0.055, and 0.55 mg / g.

Conclusion. 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone demonstrated cytotoxic effects on the A549 cell line at a lower IC_{50} than cisplatin which is widely used in treatment of cancers, including lung cancer. Insolubility of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone in water and the absence of its toxic effect in the studied modes determine the scope of its application for further study of cumulative and antitumor effects.

Keywords: tropolones, antitumor effect, human non-small-cell lung cancer A549 cell line, MTT assay

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

Source of financing. The study was carried out within the state assignment “The study of antitumor effects of pharmaceutical ingredients *in vivo* and *in vitro*” (No. 121031100253-3).

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Conformity with the principles of ethics. The study was approved by the local Bioethics Committee at the National Medical Research Center of Oncology (Protocol No. 1/61 of 19.02.2019).

For citation: Kit O.I., Minkin V.I., Lukbanova E.A., Sayapin Yu.A., Gusakov E.A., Sitkovskaya A.O., Filippova S.Yu., Komarova E.F., Volkova A.V., Khodakova D.V., Mindar M.V., Lazutin Yu.N., Engibaryan M.A., Kolesnikov V.E. Evaluation of the cytotoxic activity and toxicity of a tropolone derivative with a potential antitumor effect. *Bulletin of Siberian Medicine.* 2022;21(1):60–66. https://doi.org/10.20538/1682-0363-2022-2-60-66.

Оценка цитотоксической активности и токсичности производного тропалонов с потенциальным противоопухолевым действием

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РЕЗЮМЕ

Цель – исследование токсичности 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалона *in vitro* и *in vivo*.

Материалы и методы. Для синтеза 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалона использован метод расширения о-хинонового цикла в процессе реакции между 5-нитро-2,6,8-триметил-4-хлорхинолином и 3,4,5,6-тетрахлор-1,2-бензохиноном при кипячении в диоксане. Эксперимент *in vitro* проведен на клеточной линии рака легкого человека A549. Оценку жизнеспособности клеток проводили при помощи МТТ-колориметрического теста по уменьшению оптической плотности опытных проб по сравнению с контрольными. Исследование острой токсичности проведено на 20 самках мышей линии Balb/c Nude. Исследуемое соединение вводили однократно перорально в форме суспензии в 1%-м крахмальном геле в трех дозах: 0,0055 (1-я группа), 0,055 (2-я группа) и 0,55 мг/г (3-я группа). Контрольная группа (4-я) получала плацебо.

Результаты. Получен 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалон по ранее разработанному методу, его строение установлено данными ядерно-магнитного-резонанса ^{1}H и инфракрасной и масс-спектрометрии. Выход составил 19,8 г (52%), температура плавления 205–207 °С, ярко-желтые кристаллы (бензол). Ингибирующая концентрация IC_{50} 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалона была равна 0,21 ± 0,01 мкМ, что оказалось статистически значимо меньше ($p < 0,05$) ингибирующей концентрации IC_{50} цисплатина равной 3,84 ± 0,23 мкМ. В результате исследования *in vivo* не выявлено токсического действия тропалона при однократном введении в дозах 0,0055; 0,055 и 0,55 мг/г.

Заключение. Показано, что 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалон проявляет цитотоксическую активность в отношении клеточной линии A549 в более низкой ингибирующей концентрации IC50, чем цисплатин, широко применяющийся в лечении злокачественных новообразований, в том числе рака легкого. Нерастворимость в воде 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалона и отсутствие его токсического действия в исследованных нами режимах определяют границы его использования для дальнейшего изучения кумулятивных и противоопухолевых эффектов.

Ключевые слова: тропалоны, противоопухолевый эффект, культура клеток немелкоклеточного рака легкого человека A549, МТТ-тест

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Работа выполнена в рамках государственного задания «Изучение противоопухолевой активности фармакологических субстанций *in vivo* и *in vitro*» (№ 121031100253-3).

Соответствие принципам этики. Исследование одобрено биоэтической комиссией НМИЦ онкологии (протокол № 1/61 от 19.02.2019).

Для цитирования: Кит О.И., Минкин В.И., Лукбанова Е.А., Саяпин Ю.А., Гусаков Е.А., Ситковская А.О., Филиппова С.Ю., Комарова Е.Ф., Волкова А.В., Ходакова Д.В., Миндарь М.В., Лазутин Ю.Н., Енгибарян М.А., Колесников В.Е. Оценка цитотоксической активности и токсичности производного трополонов с потенциальным противоопухолевым действием. *Бюллетень сибирской медицины.* 2022;21(2):60–66. https://doi.org/10.20538/1682-0363-2022-2-60-66.

INTRODUCTION

In the modern world, cancer is considered one of the most severe diseases with frequent fatal outcomes [1]. In addition, low efficiency and low selectivity of cytotoxic drugs used in clinical practice are combined with many side effects [1] and a small range of effective doses [3]. The use of effective treatment methods is one of the key factors for improving the prognosis of the course of cancer [4]. Therefore, development and study of new anticancer drugs that combine high cytotoxic activity with minimal side effects remain relevant.

Along with well-known chemotherapeutic agents, some scientists and physicians propose to use certain podophyllotoxin derivatives, diterpenes, and alkaloids as medicines [5]. A promising group of substances with a wide range of biological activities, including antitumor ones, is non-benzenoid aromatic compounds – tropolones [6, 7]. Their most studied representatives are β-thujaplicin (hinoktiol), colchicine, and colchamine. There are some approaches to the synthesis of tropolones allowing to obtain a wide range of substances with various biological properties, such as antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, and antitumor ones [5, 8].

2-[7-acetyl-9,11-di(tert-butyl)-4-methyl-5-chlorobenzo[b][1,4]oxazepino[7,6,5-de]-quinoline-2-yl]-5,6,7-trichloro-1,3-tropolone has the most similar structure to the studied compound; it shows cytotoxic activity against breast tumor cells MCF-7 and MCF-10 cell line, lung cancer cells Lu, liver cancer cells Hep-G2, and tumor epithelial cells KB [6]. Derivatives of 2-quinoline-2-yl-1,3-tropolones have shown activity against various cancer cell lines in the lungs (A549 and H441), ovaries (OVCAR-3 and OVCAR-8), colon (HCT 116), and pancreas (Panc-1) in the range from $IC_{50\ to\ 5}$ μM [9].

The aim of this study was to analyze the toxicity of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone *in vitro* and *in vivo*.

MATERIALS AND METHODS

Studied compound. The structural formula of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone is presented in

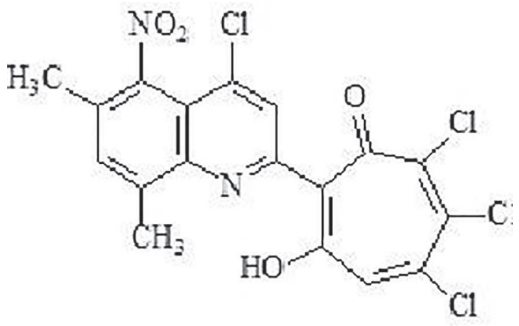

Fig. 1. Structural formula of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone

Fig. 1. Compound 1 (2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone) was synthesized using a method for expanding the *o*-quinone cycle [10]. The reaction proceeded between 5-nitro-2,6,8-trimethyl-4-chloroquinoline (2) and 3,4,5,6-tetrachloro-1,2-benzoquinone (**3**) while boiled in dioxane (Fig. 2)

Cytotoxic activity testing. The experiment was carried out in the human non-small-cell lung cancer A549 cell line. Cell viability was assessed using the MTT colorimetric assay. The cells were cultured in a 96-well plate under standard sterile conditions: temperature of +37 ºC, 5% CO2, DMEM culture medium, 10% FBS. The tested substance, the reference drug (cisplatin), and the solvent (DMSO) were added at the concentration of 0.004–2.226 μM. The cells continued to be incubated under the same conditions for 72 hours, after which 20 μL of the MTT solution

was added to them. Then the incubation was continued for another 2 hours. Formazan crystals formed as a result of the MTT assay were dissolved in DMSO, and the optical density (the average wavelength was 492 nm) was measured using the Stat Fax 2100 Microplate Reader (Awareness Technology, USA) [11].

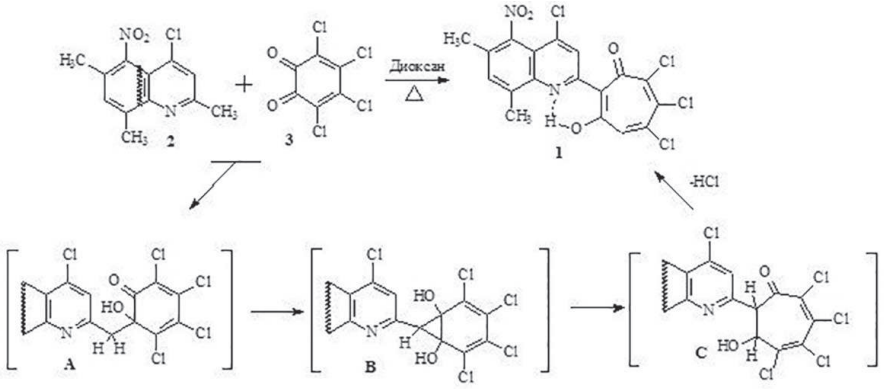

Fig. 2. Synthesis of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone

Acute toxicity testing. Acute toxicity was studied on 20 BALB/c Nude female mice weighing 25.5–27.5 g. They were divided into 4 groups of 5 animals each. The study was conducted on females because they are considered more sensitive to the effects of substances than males (GOST 32296-2013). Since the experiment included linear animals and followed the principles of the 3Rs, 5 animals were used in each group, which seemed possible for statistical analysis of the data [12].

The acute toxicity of tropolone was studied on BALB/c Nude mice, since this compound with a potential antitumor effect can be studied on xenograft models created on immunodeficient mice, which are considered more sensitive to the exposure [13, 14]. The studied compound was administered once orally as a suspension in 1% starch gel at three doses: 0.0055 mg / g (group 1), 0.055 mg / g (group 2), and 0.55 mg / g (group 3). The choice of a maximum dose was limited by the insolubility of the test compound in water. The control group (group 4) received 1% starch gel. After the administration of the substance, the animals were examined daily for 14 days. The body weight of the animals was determined on days 7 and 14. The following parameters were used to assess the dose-dependent effects of tropolone: survival rate, health disorders during daily observation, weight dynamics; at necropsy: deviations from the normal condition of the skin and visible mucous membranes, as well as deviations from normal size, shape, color, structure, and location of internal organs, body cavity effusions, and secretions from natural body orifices (GOST R 56701-2015). Animals were euthanized on day 14 of the experiment by cervical dislocation.

Statistical analysis of the data. To determine the inhibitory concentration when testing the cytotoxic activity of the compound, the proportion (%) of viable cells was calculated in the test wells relative to the positive control wells, which cell viability was considered as 100%. Logarithm of the concentration at 50% cell viability was calculated by the probit analysis, and then the half-maximal inhibitory concentration (IC50) was calculated [15]. In the statistical analysis of the results, the mean and the standard deviation $M \pm m$ were evaluated; the Wilcoxon – Mann – Whitney test and the Kruskal – Wallis test with the post-hoc Dunn's test were used. The GraphPad Prism 5.0 software was used for statistical analysis of the results.

RESULTS

2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone was obtained according to the previously developed method [16], and its structure was established by ^{1}H nuclear magnetic resonance (NMR), infrared (IR) spectroscopy, and mass spectrometry. When assessing the cytotoxicity of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone against the A549 cell culture, its IC_{50} was determined. It reached 0.21 ± 0.01 μM, which turned out to be statistically significantly lower than the IC_{50} of cisplatin equal to 3.84 ± 0.23 μM (the differences were statistically significant compared with the control group ($p < 0.05$, according to the Kruskal – Wallis test), widely used in clinical practice [17].

The analysis of the acute toxicity of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-tri-

chloro-1,3-tropolone with its single administration at the dose of 0.0055 mg / g (group 1), 0.055 mg / g (group 2), and 0.55 mg / g (group 3) showed 100% survival rate, which did not allow to detect a lethal dose. Administration of higher doses of the studied substance was impossible because the volume fraction of the solid dispersed phase exceeded that of the liquid dispersed phase, and the substance was water-insoluble. No health disorders were registered during 14 days of daily observation in all four groups of animals. Necropsy showed no pathological changes in most mice. In group 2, two mice had single focal hemorrhages up to 1 mm in diameter in the medial lobe of the liver. In group 3, two animals had hyperemia in the liver, and one animal had focal pulmonary hemorrhage. Pathological changes found at necropsy in some experimental mice could result from the toxic effect of tropolone or from euthanasia.

The analysis of the weight dynamics in mice with a single tropolone administration showed its slight decline by the end of the observation period in only one animal from group 3. In the other animals, on the contrary, an increase in weight by 0.5–2 g was observed by the end of the experiment compared with the baseline values. Such an increase was most pronounced in the control group and in group 1, where the mice received a minimum compound dose of 0.0055 mg / g. The weight dynamics in the animals of these groups was significantly different from that in group 3 with mice receiving the substance at the maximum studied dose of 0.55 mg / g (Table).

Table

Weight dynamics in BALB/c Nude mice on day 14 of the experiment after a single dose of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone, $M \pm m$

Group	Body weight, g
1, $n = 5$	1.6 ± 0.11▫
2, $n = 5$	1.3 ± 0.29
3, $n = 5$	0.8 ± 0.38*•
4, controls, $n = 5$	1.7 ± 0.14▫

Note: * differs from values in the control group, $p < 0.01$; • differs from values in group 1, $p < 0.01$, ▫ differs from values in group 3, $p < 0.01$; Wilcoxon – Mann – Whitney test.

The above intergroup differences may be associated with metabolic and other changes in the body of mice under the influence of the studied substance.

DISCUSSION

A number of studies demonstrated high antitumor effect of tropolones together with their minimal effect on normal tissues and low toxicity, which makes this group of compounds promising [18]. To date, hinoktiol (β-thujaplicin) is the most studied tropolone, which exhibits high antitumor activity against various cancer cell lines [19, 20].

L. -H. Li et al. determined the viability of A549 tumor cells exposed to hinoktiol as 52.7 ± 3.6 and 34.7 ± 5.2 when exposed for 48 hours at a concentration of 5 and 10 μM, respectively, and 28.9 ± 1.1 and 18.2 ± 7.2 when exposed for 72 hours at a concentration of 5 and 10 μM, respectively [21]. H. Wakabayashi et al. demonstrated that IC50 of synthesized compounds from the group of tropolones, 7-bromo-2(4-hydroxyanilino)-tropone and 4-isopropyl-2-(2-hydroxyanilino)-tropone, against human oral squamous cell carcinoma cell lines HSC-2, HSC-3, and HSC-4 varied from 31 to 450 μM depending on the incubation time (24, 48, 72, and 96 hours), which was significantly higher than the IC50 of the tropolone we synthesized [22]. Our results are consistent with the data of other researchers and may indicate high antitumor efficacy of a new compound, 7-bromo-2(4-hydroxyanilino)-tropone and 4-isopropyl-2-(2-hydroxyanilino)-tropone.

A number of studies explained high antitumor efficacy of hinoktiol. L. -H. Li et al. confirmed that hinoktiol induces autophagy, cell cycle arrest in the S-phase, and cellular aging in lung cancer cells and inhibits cell proliferation. Thus, hinoktiol, probably like other substances from the tropolone group, can act as an effective anticancer agent due to induction of DNA damage, autophagy, cell cycle arrest, and cellular aging [21].

A comparison of the acute toxicity of our synthesized compound with that of other analogous substances showed its minimal toxic effect on the body of laboratory animals. Thus, LD50 for γ-thujaplicin, β-dolabrin, and hinoktiol were 277 mg / kg, 232 mg / kg, and 191 mg / kg, respectively [23]. Y. Morita et al. determined LD50 for 4-acetyltropolone, hinoktiol, β-dolabrin, γ-thujaplicin, and α-thujaplicin, which are 335.2; 191; 232; 277, and 256 mg / kg, respectively [24]. In our study, the acute toxicity of 7-bromo-2(4-hydroxyanilino)-tropone and 4-isopropyl-2-(2-hydroxyanilino)-tropone was not detected.

CONCLUSION

The MTT assay showed that 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone exhibited cytotoxic activity against the human non-small-cell lung cancer A549 cell line, and

the IC50 of the proposed compound was lower than that of cisplatin.

Insolubility of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone in water limited the range of its investigated doses and did not allow for determination of its lethal dose and toxicity class while studying the acute toxicity. The absence of pronounced toxic effect of tropolone administered at a single dose of 0.0055, 0.055, and 0.55 mg / g determines the scope of its use for further study of cumulative and antitumor effects.

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Acknowledgments

The authors thank Olesya A. Ossovskaya, translator, for the help with the manuscript translation in English.

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Kit O.I. – study design, interpretation and analysis of the results. Minkin V.I. – conception and design, synthesis of the substance. Lukbanova E.A. – interpretation and analysis of the results, drafting of the manuscript. Sayapin Yu.A. – synthesis of the substance, drafting of the manuscript. Gusakov E.A. – carrying out of 1H NMR and IR spectroscopies, mass spectrometry, analysis of the results. Sitkovskaya A.O. – carrying out of MTT assay, editing of the manuscript. Filippova S.Yu. – working with the cell culture, carrying out of MTT assay. Komarova E.F. – conception and design, editing of the manuscript. Volkova A.V. – carrying out of a probit analysis. Khodakova D.V. – statistical analysis. Mindar M.V. – drafting of the manuscript. Lazutin Yu.N. – editing of the manuscript. Engibaryan M.A. – technical editing of the manuscript. Kolesnikov V.E. – drafting of the manuscript, compilation of the references.

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Received 23.05.2021;
approved after peer review 30.09.2021;
accepted 05.10.2021