Solubilized Formulations of Triphenylphosphine Derivatives of Allylbenzenes and Their Potential as Individual Antitumor Agents or Adjuvants to Paclitaxel

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Article

Solubilized Formulations of Triphenylphosphine Derivatives of Allylbenzenes and Their Potential as Individual Antitumor Agents or Adjuvants to Paclitaxel

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Abstract: Allylbenzenes (apiol, dillapiol, myristicin and allyltetramethoxybenzene) are individual components of plant essential oils that demonstrate antitumor activity, and can enhance the antitumor activity of cytotoxic drugs, such as paclitaxel, doxorubicin, cisplatin, etc. Triphenylphosphine (PPh3) derivatives of allylbenzenes are 2-3 orders of magnitude more potent than original allylbenzenes in terms of IC50. Inhibition of efflux pumps has been reported for allylbenzenes, and PPh3 moiety is deemed to be responsible for preferential mitochondrial accumulation and depolarization of mitochondrial membranes. However, due to poor solubility the practical use of these substances has never been an option. Here we show that this problem can be solved by cyclodextrin-based (CD) molecular containers, which are able to solubilize both allylbenzenes and their PPh3-derivatives. Further, we have observed that solubilized PPh3-derivatives indeed work as adjuvants, increasing the antitumor activity of paclitaxel against adenocarcinomic human alveolar basal epithelial cells A549 by an order of magnitude (in terms of IC50), in addition to being quite powerful cytostatics themselves. Even more important, the CD-solubilized PPh3-derivatives show pronounced selectivity, being highly toxic for tumor cells line (A549), and minimally toxic for non-tumor cells HEK293T, red blood cells, and sea urchin embryos. In many cancers, mitochondrial membrane is more prone to depolarization, as compared to normal cells, which prob-ably explains the observed selectivity of our compounds, since PPh3-derivatives are known to act as mitochondria-targeting agents.

Keywords: PPh_3 conjugates; allylbenzenes; apiol; synergism; A549; efflux inhibitor; anticancer activity

1. Introduction

Modern therapeutic strategies are in some cases ineffective against bacterial infections and cancers, which is most often associated with multiple drug resistance (MDR) [1–11]. Resistance mechanisms that reduce the likelihood of a patient's cure can be divided into two groups [12]: i) cellular metabolism (transferases, topoisomerases, growth factors), which alter the mechanism of action of drugs or interfere with their action, ii) decrease in intracellular concentration of the drug. The drug enters the intracellular medium through the transport channels of the plasma membrane, on which pump proteins (ATP-binding cassette protein [5,13]) can be expressed, pumping the drug into the extracellular medium, reducing its effect [5,6,14–17]. The main member of the efflux pump family, MDR1 (P-glycoprotein [4,13,14,18]), causes resistance of various types of tumors to

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chemotherapy. Bacteria also have efflux pumps (for example, NorA [5,10,19–21], P-glycoprotein) which cause the ineffectiveness of antibiotics. A number of substances that inhibit efflux (verapamil, reserpine, etc [22]) are known to be rather toxic. Therefore, numerous studies are aimed at finding substances that inhibit efflux, but at the same time are non-toxic.

Currently, a promising and promising direction is the use of medicines based on components of natural extracts and oils [23–43] – usually to strengthen the main drug (antibacterial or antitumor drug) and reduce the therapeutic load on the body. Individual components of essential oils (allylbenzenes [44–47], terpenoids [48,49], terpenes [50–52], flavonoids [41,53,54], Thai herbs [55], etc.) have antioxidant, antibacterial, restorative and antitumor properties, and moreover, they are effective inhibitors of efflux pumps [4,6,7,10,14–17,20,21,39,56–60] that cause bacterial resistance to antibiotics and resistance of cancer cells to cytostatics. Thus, individual components of essential oils and their modifications are potential candidates for powerful medicinal combinations. However, such substances are often lipophilic [2,3,19,35,38,50,58,61–64], which makes it difficult to use them in medical practice, so the adjuvant should be used in a molecular container such as liposomes or polymeric carrier. Cyclodextrins (CD) [23,42,43,65–76] or polycations/polyanions (chitosan, polyethyleneimine, pectin, alginate, heparin, etc) can serve as effective solubilizing containers, that improve the bioavailability and pharmacological properties of the drug.

Apiol (1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene) is an component of parsley oil inhibits cytochrome P450 3A4 (IC_{50} 7.9 μM) [19,28,44,58,77–80], which metabolizes xenobiotics in the liver, reducing their bioavailability. Apiol demonstrates weak antibacterial and anticancer activities, but at the same time dramatically enhances the effect of antibiotics (for example, moxi-, levofloxacin) [44,77] and cytostatics (doxorubicin, paclitaxel, etc.) [49] by inhibiting P-glycoprotein.

Apiol analogues (myristicin, allyltetramethoxybenzene and dillapiol) also demonstrated weak antitumor activity, but showed an increase in the main component of the antitumor formation (paclitaxel, doxorubicin, cisplatin) [49] due to inhibition of mitochondrial enzymes [81,82], efflux pumps [49] and increased permeability of the membrane of cancer cells [49,83]. It was previously shown that dillapiol (25-50 μM) induced G0/G1 cell cycle arrest, activation of a number of caspases and, accordingly, apoptosis of cancer cells, while apiol and analogues had virtually no effect on benign epithelial cells *in vitro* [46]. Myristicin showed a similar but weaker effect. Recently triphenylphosphine (PPh_3) derivatives of allylbenzenes were suggested as an approach to improve their antiproliferative potency towards cancer cells taking into account their tendency to preferential mitochondrial accumulation [46]. The introduction of a hydrophobic charged fragment optimizes the location of the conjugate in the cell membrane and increases the inhibitory ability against mitochondrial membrane enzymes (previously shown by us on the micellar model) [82]. Cancer cells have altered metabolism, in particular the dynamics of mitochondria (the PPh_3 fragment can serve as an address label to cancer mitochondria), which provides many potential targets for cancer therapy [84–86].

Considering the mechanism of PPh_3-derivatives of allylbenzenes action, we can assume their potential synergistic effect with the main drug, paclitaxel. The mechanism of action of Paclitaxel is based on the suppression of the normal process of dynamic reorganization of the microtubule network, responsible for cell division. In addition, paclitaxel induces the formation of abnormal clusters and causes the formation of multiple microtubule stars during mitosis. Paclitaxel is used as a first-line drug in the treatment of ovarian, breast, lung, cervical cancer, etc. The combination of paclitaxel + adjuvant is expected to be more effective than a single drug due to the action of different mechanisms and an increase in the bioavailability of cytostatic.

In this paper, the key idea is to realize three main aspects to create an enhanced antitumor formation: i) a combination of the main cytostatic (Paclitaxel) with an adjuvant (as efflux inhibitor) from the group of allylbenzenes, ii) an increase in the mitochondrial bioavailability of the adjuvant by conjugating it with a PPh_3 fragment due to depolarization of mitochondrial membranes in cancer cell, iii) the use of cyclodextrins derivatives and heparin polysaccharide matrix as molecular containers to obtain soluble forms of drugs and increases in bioavailability.

2. Materials and Methods

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2.1. Reagents

γ-cyclodextrin (γ-CD), methyl β-cyclodextrin (M-β-CD) were purchased from Sigma Aldrich (St. Louis, MI, USA). Apiol, dillapiol, allyltetramethoxybenzene and myristicin were isolated from plant extracts as described earlier [77]. Heparin (MM 50-80 kDa), organic solvents, salts and acids were Reakhim (Moscow, Russia) production.

The synthesis of triphenylphosphine derivatives of allylbenzenes was performed as described earlier in the work [46]. The ^{1}H and ^{13}C NMR spectra of the Apiol-PPh3 and analogues in d_6-DMSO were recorded on a Bruker Avance 400 spectrometer (Bruker Biospin, Rheinstetten, Germany) at an operating frequency of 400 MHz. The chemical shifts are shown in ppm on the δ scale relative to hexamethyldisiloxane as an internal standard. The analysis and processing of the NMR spectra were performed with the program MestReNova v.12.0.0–20080).

2.2. Non-covalent complexes of Apiol-PPh3 and analogues with cyclodextrins and heparin

Non-covalent complexes of Apiol-PPh3 and analogues with cyclodextrins (1:2 mol/mol) and heparin (15 kDa, 1:1 w/w) were obtained by adding of solutions of cyclodextrins and heparin in PBS to Apiol-PPh3 (and analogues) samples to achieve various excesses of oligo and polymers, followed by incubation for 1 hour at 40 °C. CD spectra of heparin were recorded on the Jasco J-815 CD Spectrometer (Japan) for the determination of heparin in the tested formulations. Concentrations of the active substance varied from 10^{-2} to 10^{-4} M. For experiments by dilution in a cell growth medium or buffer substances in the concentration range from 10^{-3} to 10^{-9} M were studied.

2.3. Determination of the dissociation constants of complexes of Apiol-PPh3 and analogues with cyclodextrins and heparin

ATR-FTIR spectra of samples (Sections 2.2) were acquired using a Bruker Tensor 27 spectrometer equipped with a liquid N_2 cooled MCT (mercury cadmium telluride) detector. Samples were placed in a thermostatic cell BioATR-II with ZnSe ATR element (Bruker, Germany). FTIR spectra were recorded from 850 to 4000 cm^{-1} with 1 cm^{-1} spectral resolution, 50 scans were accumulated and averaged. Spectral data were processed using the Bruker software system Opus 8.2.28 (Bruker, Germany). The spectrum of cyclodextrin or heparin in the corresponding concentration was subtracted from the spectra of the complexes. Then the dependences of the peak intensities of the corresponding C=C oscillation (aromatic system of apiol-PPh3 and analogues (1475-1510 cm^{-1})) was constructed, which least overlaps with the spectrum of cyclodextrin and heparin.

Calculation of the dissociation constants X – M-β-CD, X – γ-CD and X – heparin, where X is Apiol-PPh3 and analogues, was performed as follows:

1) consider the equilibrium (given for the M-β-CD, for the rest it is the same): $X + nM\text{-}\beta\text{-}CD \leftrightarrow X \cdot nM\text{-}\beta\text{-}CD$, where $K_d = [M\text{-}\beta\text{-}CD]^n \cdot [X] / [X \cdot nM\text{-}\beta\text{-}CD]$;

2) Complexation degree calculation $\theta = (\xi - \xi_0) / (\xi_\infty - \xi_0)$, where ξ is FTIR peak current intensity, ξ_0 is FTIR peak initial intensity (only Apiol-PPh3 and analogues without M-β-CD, etc), ξ_∞ is FTIR peak intensity of Apiol-PPh3 and analogues with a large excess of M-β-CD, etc;

3) Linear fitting of data: $\lg(\theta / (1 - \theta))$ versus logarithm of concentration of the M-β-CD, γ-CD or heparin was carried out using the Hill equation: $\lg(\theta / (1 - \theta)) = n \cdot \lg[M\text{-}\beta\text{-}CD] - \lg K_d$.

2.4. Cell cultivation and determination of cytotoxic activity

Adenocarcinomic human alveolar basal epithelial cells A549 cell lines (Manassas, VA, USA) were cultured in RPMI-1640 medium, linear cells of the embryonic kidney human epithelium (HEK293T) were grown in DMEM medium as described earlier [49]. Cell lines were obtained from Lomonosov Moscow State University Depository of Live Systems Collection and Laboratory of Medical Biotechnology, Institute of Biomedical Chemistry (Moscow, Russia).

Cytotoxic activity of Paclitaxel, Apiol-PPh3 and analogues was determined using MTT test [49]. Paclitaxel-adjuvant synergism coefficients (SC) were calculated as CV(paclitaxel)×CV(alone adjuvant) / CV(combo Paclitaxel+adjuvant), where CV is cell viability. Synergy coefficient can be

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interpreted as strong synergy (SC>2), synergy (2>SC>1.2), indifference/additivity (1.2>SC>0.8), antagonism (0.8>SC>0.5), inhibition (SC<0.5) – as earlier described [49,77].

2.5. Phenotypic Sea Urchin Embryo Assay

Adult seaurchins, *Paracentrotus lividus L.* (Echinidae), were collected from the Mediterranean Sea on the Cyprus coast and kept in an aerated seawater tank and were used to study cleavage alteration of Apiol-PPh_3 and analogues [46,87]. Experiments with sea urchin embryos comply with the requirements of biological ethics. Artificial spawning does not lead to the death of animals, embryos develop outside the female body, and both adult sea urchins after spawning and an excess of intact embryos return to the sea, their natural habitat.

2.6. Study of the safety of formulations (hemolytic activity and thrombogenicity)

Hemolytic activity and thrombogenicity of Apiol-PPh_3 and analogues were studied using earlier published technique [83].

2.7. Statistical Analysis

Statistical analysis of cytotoxicity and spectral data was performed using the Student's t-test Origin 2022 software (OriginLab Corporation). Values are given as the mean ± SD of three or five experiments.

3. Results and Discussion

3.1. Article Design

The present work is aimed at developing and studying complex antitumor formulations based on three components: the main drug (paclitaxel), adjuvant (apiol-PPh_3 and its analogues), molecular container (cyclodextrins (CD) for the formation of inclusion complexes with paclitaxel and its adjuvant in the hydrophobic cavity of CD or heparin polyanion to stabilize the cationic triphenylphosphine fragment). In previous studies, we have shown that cytostatics (paclitaxel, doxorubicin, etc.) are enhanced by allylbenzenes, which can also act as promising anticancer drugs [49,88]. In this paper, PPh_3-derivatives of allylbenzenes are used to enhance the effect due to depolarization of mitochondrial membranes and taking into account their tendency to preferential mitochondrial accumulation. However, PPh_3-derivatives are poorly soluble, so to realize their potential, it is required to develop the optimal container, providing an increase in allylbenzene-PPh_3 solubility, obtaining double-drug inclusion complexes, which can provide synergism of the action of the main antibiotic and adjuvant (cyclodextrin derivatives or anionic polysaccharides are proposed in this work). To achieve this, the following tasks are realized: 1) spectral characterization of PPh_3-derivatives of allylbenzenes and solubility studying, 2) characterization of double-drug inclusion complexes of these compounds and paclitaxel with various cyclodextrins derivatives or heparin, determination of dissociation constants of complexes, 3) cytotoxic activity of the cytostatic agents alone and in the complex drug formulations against A549 using MTT test, 4) selectivity of the cytostatic activity and the safety of drugs for non-cancer cells HEK293T *in vitro* using FTIR spectroscopy, red blood cells and sea urchin embryos *in vivo*.

3.2. The spectral characteristics of PPh_3-derivatives of allylbenzenes

Allylbenzenes (apiol, myristicin, etc.) have a number of important biological activities, including experimental prerequisites to be synergists (enhancers) of the action of cytotoxic drugs. To increase the bioavailability of allylbenzenes, the modified form of allylbenzenes with PPh_3 fragment (Figure 1) was obtained according to the methodology described recently [46]. Confirmation of the success of synthesis follows from NMR and FTIR spectroscopy data (Figure 2, Figure S1, Table 1). The original substances (apiol and analogues) are characterized by the main signals: aromatic protons (6 and 6.5 ppm), protons at the double bond of the allyl group (5 ppm), protons of methoxy groups and/or

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methylene bridges (3.3-4 ppm). After the introduction of the PPh_3 residue into these molecules, the proton signals of the allyl group double bond disappear, but the proton signals of phenyl substituents (7.6-8.1 ppm), as well as the alkyl spacer (1.7-3 ppm) appear. In the FTIR spectra (Figure 2c) of the initial allylbenzenes, the most significant are the oscillation bands C=C 1660 cm^{-1} (allyl group) and 1450-1550 cm^{-1} (aromatic system). After modification of allylbenzenes with PPh_3, the peak of oscillations of the C=C allyl group disappears, but peaks corresponding to the deformation fluctuations of the C–H triphenylphosphine fragment (1400-1480 cm^{-1}) and fluctuations of C=C bonds (1500-1600 cm^{-1}) appear.

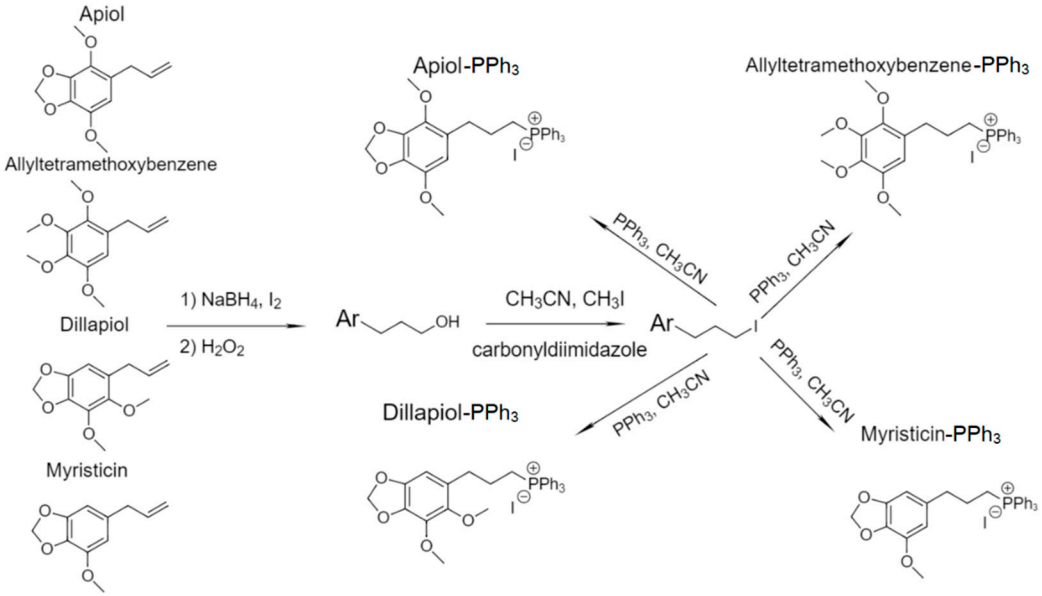
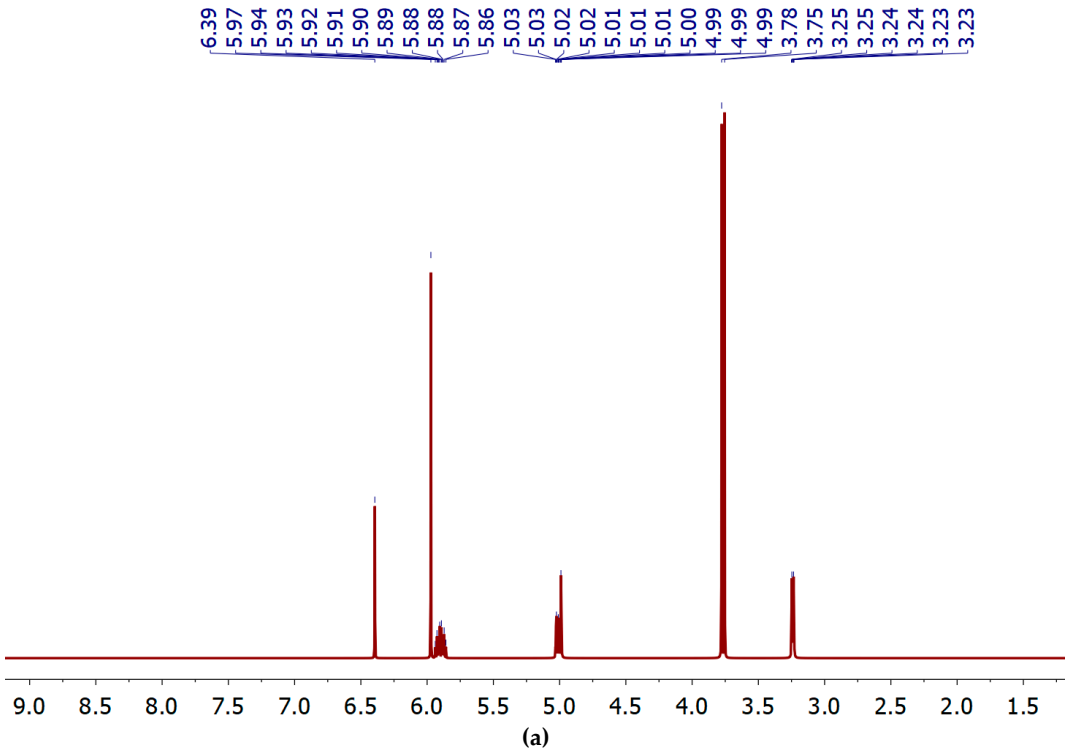


Figure 1. The scheme of synthesis of allylbenzenes' PPh_3-derivatives.



(a)

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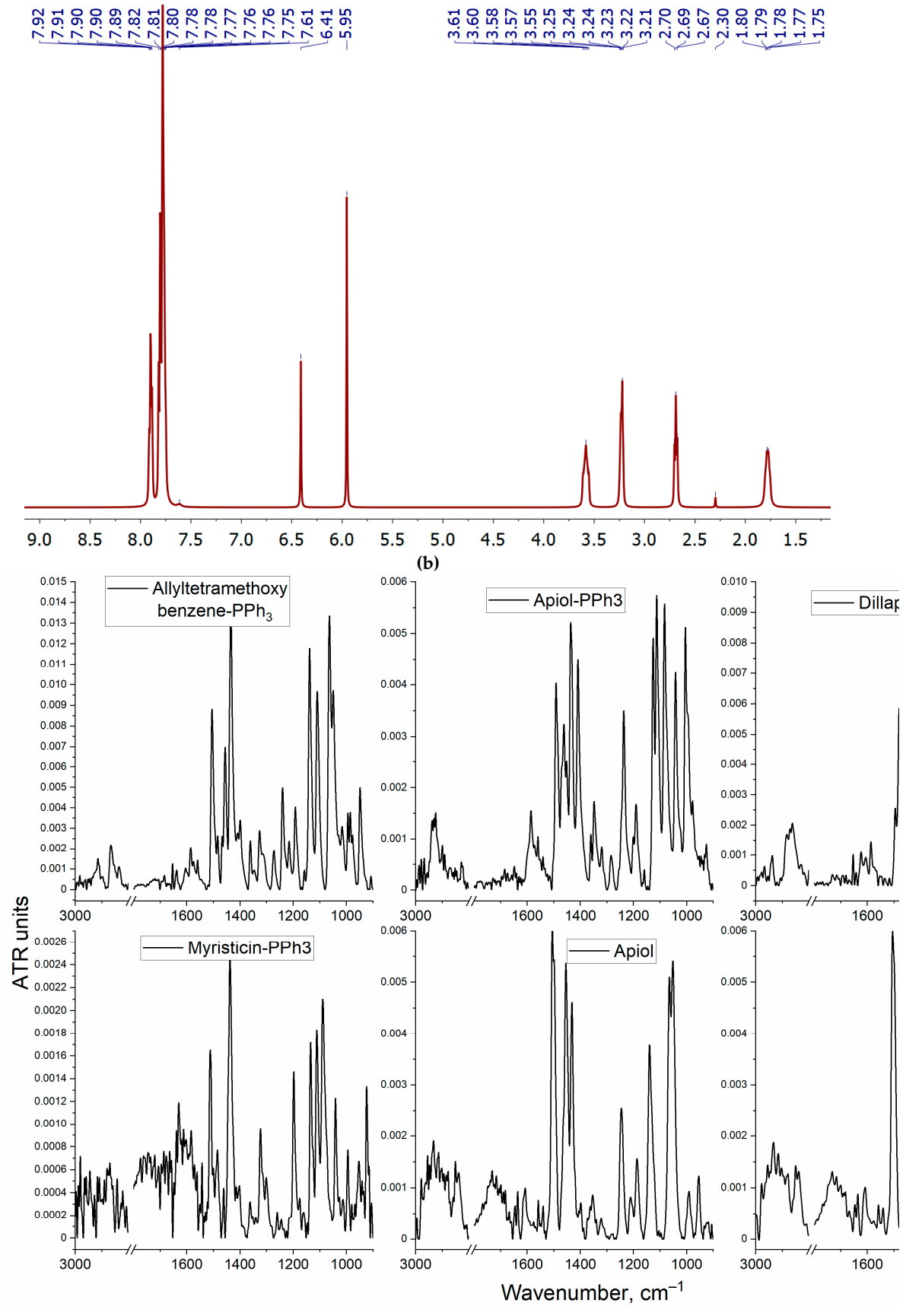

Figure 2. ^{1}H NMR spectra of (a) apiol, (b) apiol-PPh$_3$. T = 25 °C. d$_6$-DMSO. 400 MHz. (c) FTIR spectra of apiol, apiol-PPh$_3$, dillapiol-PPh$_3$, myristicin, myristicin-PPh$_3$ and allyltetramethoxybenzene-PPh$_3$. PBS. T = 22 °C.

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Table 1. Positions of characteristic peaks in the FTIR spectra of dillapiol, allyltetramethoxybenzene, PPh_3, dillapiol-PPh_3, allyltetramethoxyallylbenzene-PPh_3. For its analogues, the correlations are similar.

Compound	Functional group	Position of the characteristic peak in the FTIR spectra, cm^{-1}	
		octane-ethanol (50:50 v:v)	water-ethanol (50:50 v:v)
Dillapiol	O–$\underline{CH_2}$–O	2917	2924
	=C–O–C	1065	1045
	–O–$\underline{CH_3}$	2848	2858
	C–C aromatic	1464	1448
Allyltetramethoxybenzene	Aryl–$\underline{CH_2}$–CH=CH_2	2956	2930
	–O–$\underline{CH_3}$	2924	2901
	C–C aromatic	1492 и 1466	1488 и 1449–1456
Propyl-PPh_3	C–C aromatic	1421	1414–1420
		1440 и 1455	1455
Dillapiol-PPh_3	O–$\underline{CH_2}$–O	2937–2952	2927–2932 (2928)
	=C–O–C	1082–1087	1086 (1088)
	–O–$\underline{CH_3}$	2848	–
	Aryl–$\underline{CH_2}$–$\underline{CH_2}$–$\underline{CH_2}$–PPh_3	2970	2981 (2974)
	C–C aromatic	1502	1485
		1455 и 1465	1448-1457
Allyltetramethoxybenzene-PPh_3	=C–O–C	1086	1089 (1088)
	–O–$\underline{CH_3}$	2855	2900 (2880–2900)
	Aryl–$\underline{CH_2}$–$\underline{CH_2}$–$\underline{CH_2}$–PPh_3	2993 и 2957	2980 (2974)
	C–C aromatic	1467	1482–1488 (1486)

3.3. Solubility of PPh3-modified allylbenzenes adjuvants and complex formation with cyclodextrins and heparin

Loading both the main cytostatic agent and its adjuvants (apiol-PPh_3 and its analogues) into molecular containers is suggested as perspective approach to increase the solubility of substances in aqueous solutions and increase the bioavailability, and consequently, the effectiveness of the antitumor formulation. Previously, we studied allylbenzenes as independent antitumor preparations and adjuvants to paclitaxel, for which we obtained soluble forms due to complexation with M-β-CD [77] (otherwise, these substances cannot be used at all due to insolubility and oil-water phase separation) – Table 2. We suggested to use cyclodextrins or non-cyclic polysaccharide (as a control polymer) for the preparation of soluble formulations of triphenylphosphine derivatives. Here we consider M-β-CD, which has demonstrated a good approach for allylbenzenes solubilization, as well as γ-CD, which has a larger size of the inner cavity. We have chosen heparin as a polyanion to form electrostatic complexes with positively charged PPh_3. In addition, heparin as an antithrombotic agent in the tumor microenvironment could have additional therapeutic effect, since the tumors development is a thrombosis-associated process.

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Table 2. Dissociation constants of complexes of adjuvants and cyclodextrins or heparin. Solubility of X and X-PPh_3 in PBS and solubility of their complexes with M-β-CD in PBS. Comparison of unmodified "apiols" and the PPh_3-derivatives.

Substance **X-PPh_3**	–lg K_d (X – M-β-CD)*	–lg K_d (X – γ-CD)**	–lg K_d (X – heparin)***	Solubility in PBS, mM	Solubility in the presence of 0.05 M MCD, mM
Apiol-PPh_3	2.9±0.3	1.2±0.2	2.7±0.2	0.08 ± 0.01	15 ± 2
Dillapiol-PPh_3	2.6±0.2	1.4±0.3	3.0±0.3	0.09 ± 0.01	8 ± 1
Myristicin-PPh_3	3.0±0.3	1.3±0.1	2.6±0.2	0.04 ± 0.005	12 ± 3
Allyltetramethoxybenzene-PPh_3	3.1±0.2	2.1±0.2	3.2±0.1	0.07± 0.01	17 ± 5
Substance **X**	–lg K_d (X – M-β-CD)****			Solubility in PBS, mM	Solubility in the presence of 0.05 M MCD, mM
Apiol	2.6±0.3			0.13 ± 0.01	22 ± 4
Dillapiol	2.7±0.5			0.24 ± 0.05	27 ± 3
Myristicin	3.5±0.2			0.030 ± 0.007	41 ± 5
Allyltetramethoxybenzene	3.4±0.3			0.16 ± 0.02	38 ± 2

* The complex with M-β-CD is formed in a molar ratio of 1 to 1. ** The complex with γ-CD is formed in molar excess of **X** approximately 1.2-1.4. *** Dissociation constants were calculated per one unit of heparin by the formula $C_{12}H_{19}NO_{20}S_3$. ****Data from paper [77].

FTIR spectroscopy provides valuable data on the interaction of molecules, including those applicable to the description of non-covalent apiol-PPh_3 and its analogues complexes with cyclodextrins and heparin. In the FTIR spectra of apiol-PPh_3 and its analogues (Figure 3a) characteristic are the bands of valence oscillations of the bonds C=C of the aromatic system (1450-1650 cm^{-1}) overlapping with the bands of deformation oscillations C-H (1400-1500 cm^{-1}). The intensity of these peaks increases with the formation of non-covalent apiol-PPh_3 and its analogues complexes with cyclodextrins and heparin due to the transition of the solid phase into solution. Linear fitting of the intensity of peaks in the FTIR spectra on the concentration of cyclodextrin or heparin (Section 2.3) in Hill coordinates allows us to calculate the dissociation constants of complexes (Table 2). The interactions of triphenylphosphine derivatives of allylbenzenes with γ-CD is rather weak (K_d 10mM values). In the case of M-β-CD the dissociation constants reach millimolar values, which is sufficient to obtain soluble forms of adjuvants (Table 2). Thus, the β-cyclodextrin derivatives that are more suitable in terms of size for inclusion of the adjuvants studied. Heparin forms rather strong complexes due to multipoint electrostatic interactions: K_d 10^{-3}-10^{-4} M per heparin unit, or 10^{-5} M per heparin molecule. Comparing the values of the dissociation constants of alkylbenzenes and their triphenylphosphine derivatives complexes with cyclodextrins, we observed that these K_d values are close, which means that it is the allylbenzene-fragment (of apiol-PPh_3) that plunges into the cyclodextrin cavity, and the triphenylphosphine - radical looks outward (Figure 3b), which would provide the implementation of mitochondrial targeting of the developed formulations.

Figure 3c shows the UV spectra of myristicin-PPh_3, apiol-PPh_3 and their complexes with M-β-CD: triphenylphosphine derivatives due to their low solubility in water do not have a clearly defined spectrum, on the contrary, their complexes with MCD are highly soluble and a clear peak in the UV spectrum is pronounced (225 nm). Visually, the dissolution of triphenylphosphine derivatives of allylbenzenes is observed in a microscope (Figure 3d-g): with an increase in the molar excess of cyclodextrin, an increasing number of inclusion complexes are formed and, consequently, solubility increases (1:1 molar ratio) and crystals decrease to complete dissolution (10-fold molar excess of M-β-CD).

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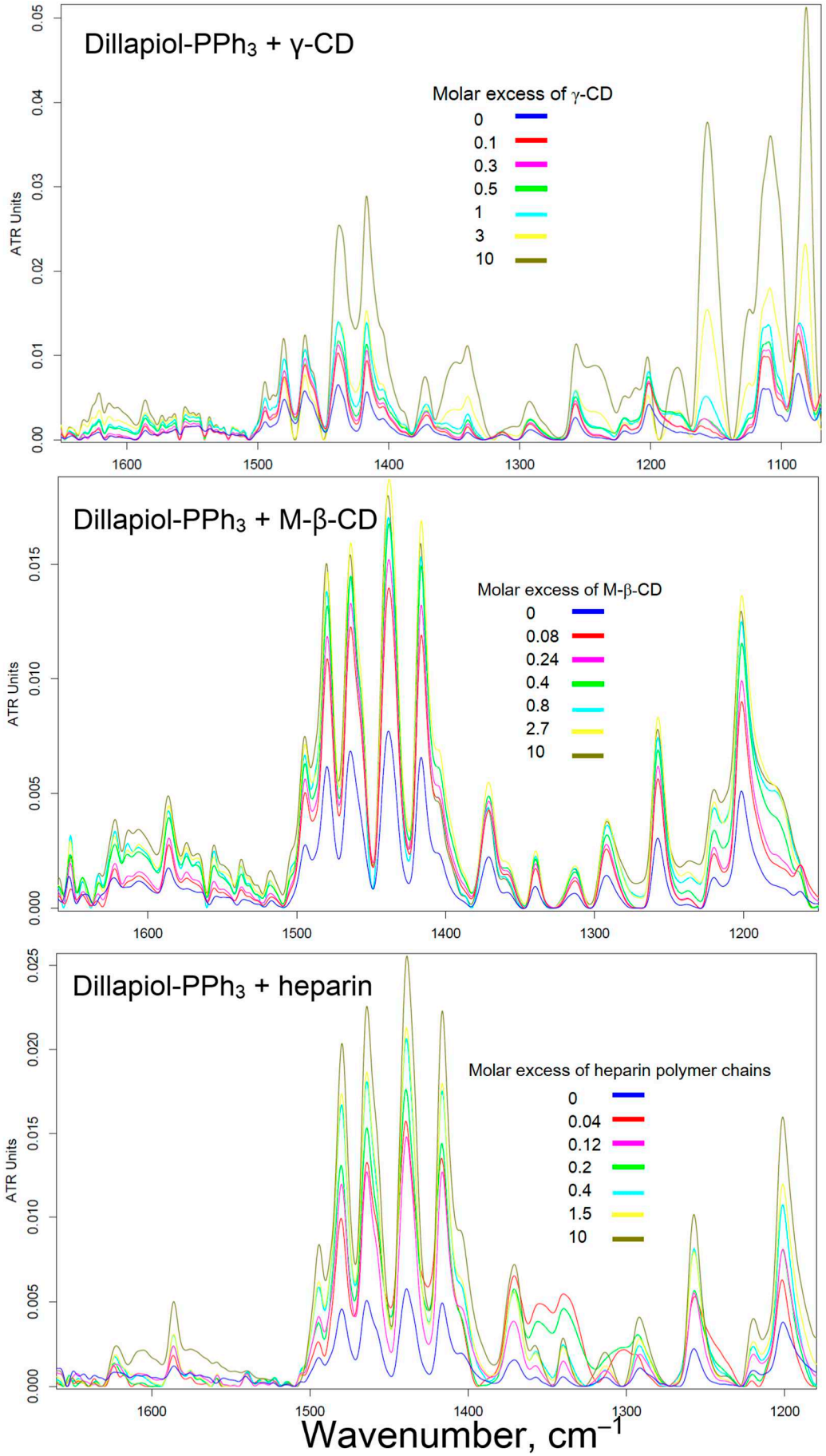

(a).

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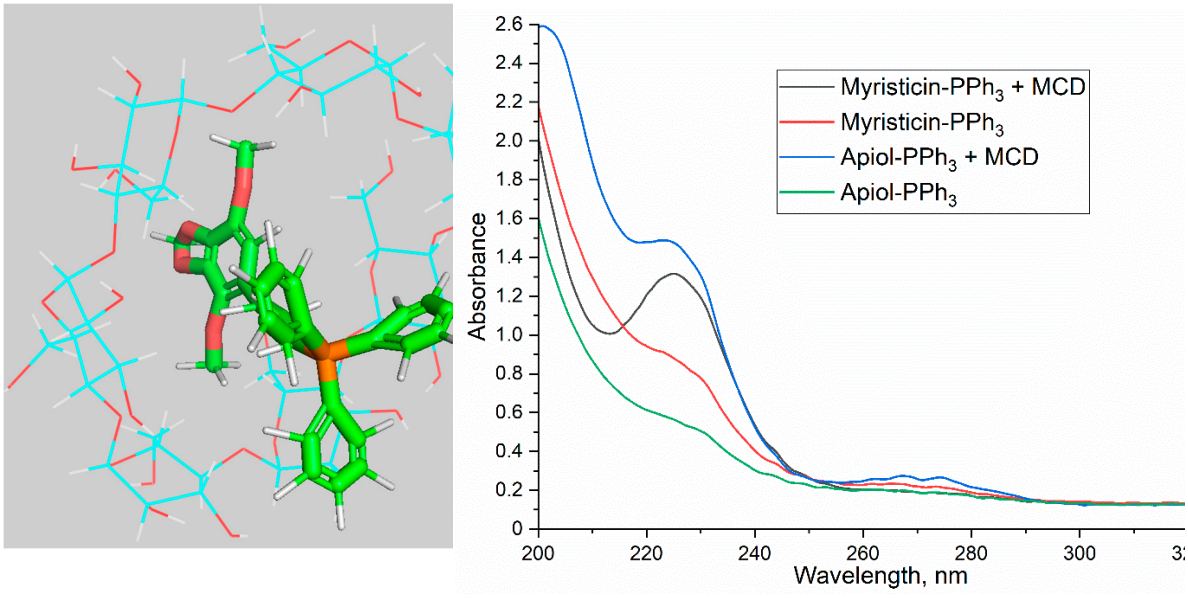
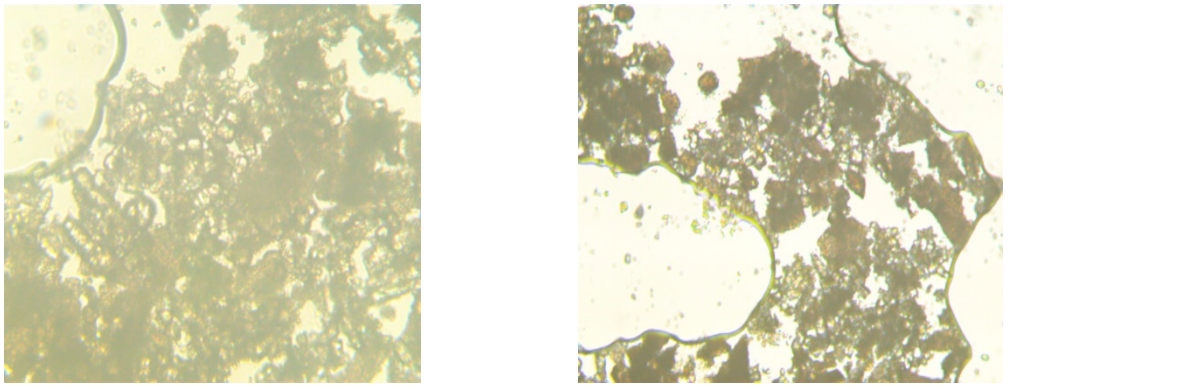
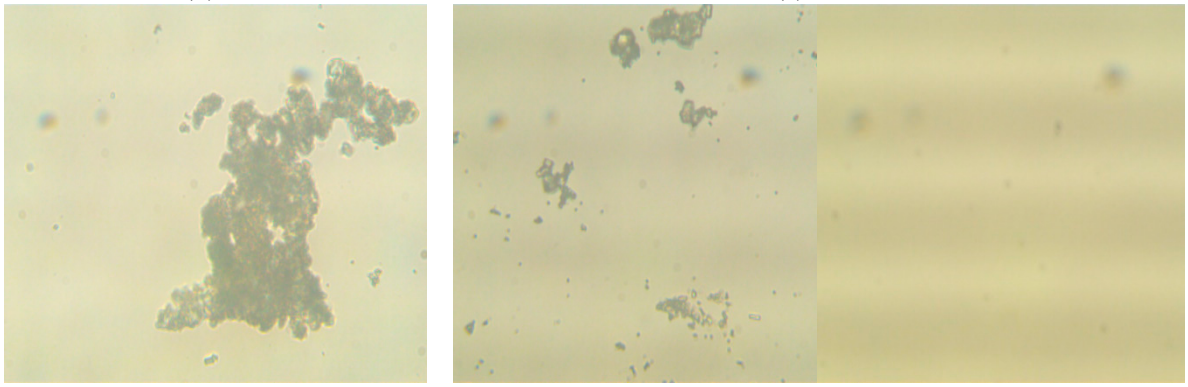


(b) (c)



(d) (e)



(f) (g) (h)

Figure 3. (a) FTIR spectra of dillapiol-PPh3 with γ-CD, M-β-CD and heparin. PBS (0.01 M, pH 7.4). T(incubation) = 40 °C. T(registration) = 22 °C. **(b)** The proposed structure of the β-cyclodextrin complex with apiol-PPh3 (for other compounds, the structure is similar). **(c)** UV spectra of myristicin-PPh3, apiol-PPh3 and the complexes with M-β-CD. **(d)-(h)** Micrographs of samples of apiol-PPh3 and its complexes with M-β-CD in the molar ratio from 1:0, 1:0.25, 1:1, 1:3 to 1:10 in given orders.

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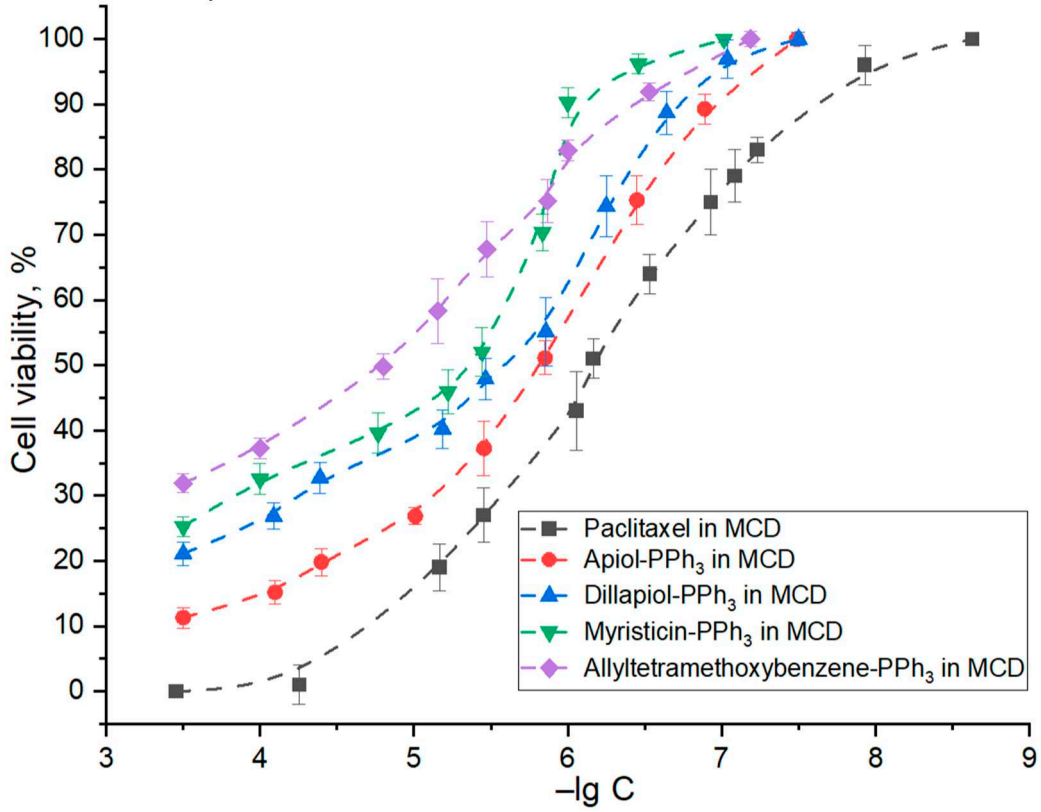
3.4. Anticancer activity of PPh_3-derivatives and formulations

Previously, we demonstrated the antitumor activity of apiol, eugenol and their analogues from the allylbenzene class, and showed the ability of these substances to act as efflux pump inhibitors and membrane-penetrating enhancer agent [49]. Apparently triphenylphosphonium derivatives of allylbenzenes effectively penetrate into cancer cells along a potential gradient, inhibit efflux proteins and mitochondrial enzymes, causing apoptosis of cancer cells by 2 orders of magnitude in smaller concentrations (Table 3). Surprisingly, PPh_3-derivatives of allylbenzenes (especially apiol-PPh_3) in the complex with M-β-CD are close in strength to the well-known cytotoxic drug paclitaxel (Figure 4a, Table 3), in addition, they demonstrate synergy with paclitaxel, by 2 order (in terms of cell viability) enhancing each other's activity. For allylbenzenes, a synergy close to additivity (the cytostatic effect of adjuvant+paclitaxel is almost equal to the sum of their individual contributions) was observed, for triphenylphosphine derivatives, a pronounced increase in the action of paclitaxel is characteristic (the cytostatic effect of adjuvant + paclitaxel is much higher (>) than the sum of their individual contributions). For apiol-PPh_3, the most pronounced effect of increasing the activity of paclitaxel was observed (Figure 4b). Thus, allylbenzene-PPh_3 inclusion complexes with cyclodextrin are potentially applicable in medicine as antitumor drugs. At the same time, it is important to find out the selectivity of the cytostatic action of the formulation developed against cancer cells and safety of these formulations for healthy cells.



(a)

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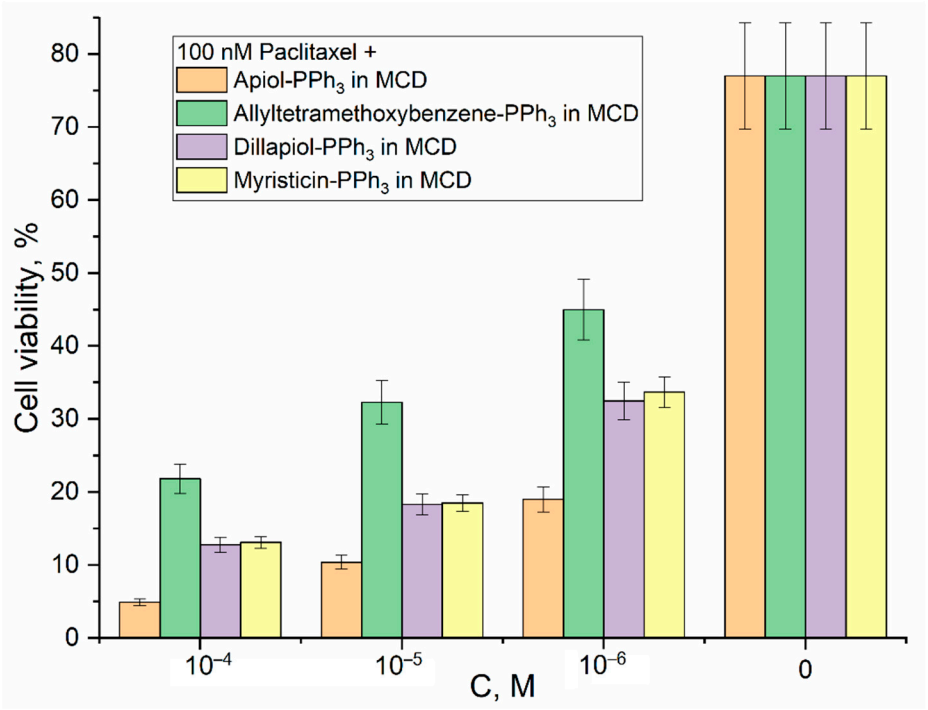

Figure 4. (a) Dependences of A549 cell viability on the concentration of Paclitaxel and allylbenzenes' PPh_3-derivatives in the form of complexes with MCD. (b) MTT assay. Dependences of A549 cell viability on the concentration of allylbenzenes' PPh_3-derivatives in the form of complexes with MCD in combinations with 100 nM Paclitaxel.

Table 3. Anti-A549 activity of PPh_3-derivatives of allylbenzenes alone and combined with paclitaxel in MCD.

Substance **X** in M-β-CD	–lg (IC50) against A549	Synergy coefficients of PPh_3 – adjuvants with paclitaxel*
Paclitaxel	6.2±0.2	-
Apiol-PPh_3	5.8±0.1	2.2±0.2
Dillapiol-PPh_3	5.6±0.2	1.5±0.1
Myristicin-PPh_3	5.3±0.2	1.8±0.3
Allyltetramethoxybenzene-PPh_3	4.8±0.1	1.3±0.1
Apiol	3.6±0.3	1.3±0.2
Dillapiol	3.2±0.1	1.1±0.1
Myristicin	2.9±0.3	0.9±0.2
Allyltetramethoxybenzene	3.5±0.2	1.4±0.2

* X – M-β-CD was studied. Synergy coefficient (SC) can be interpreted as strong synergy (SC>2), synergy (2>SC>1.2), indifference/additivity (1.2>SC>0.8), antagonism (0.8>SC>0.5), inhibition (SC<0.5). For all the studied compounds, the difference between the cytostatic effect of a combination of two substances is statistically significantly different from the effects of single substances: $p < 0.01$.

3.5. Selectivity of Action and Safety of Cytotoxic Formulations Developed

3.5.1. HEK293T as normal cell model

HEK293T are model normal (non-cancer) cells, widely use to compare the selectivity of cytostatic formulations on cancer cells [49]. Quantitatively, resulting data on safety and selectivity of action, the formulation based on triphenylphosphine derivatives is presented in Table 4. According to the MTT

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test, the concentration of cytostatics of 100 μM causes the death of up to 85% of cancer cells A549 (Figure 4a), while for non-cancer cells (HEK293T) the death is only 15-20%.

Table 4. Anti-HEK293T activity of PPh_3-derivatives of allylbenzenes in MCD as a criterion for the safety of medicinal formulations. MTT assay. RPMI-1640 medium supplemented with 5% fetal bovine serum and 1% sodium pyruvate at 5% CO_2/95% air in a humidified atmosphere at 37 °C.

Substance **X** in M-β-CD	HEK293T viability (%) at C_X = 300 μM	HEK293T viability (%) at C_X = 100 μM	HEK293T viability (%) at C_X = 10 μM
Apiol-PPh_3	71±2	82±3	93±2
Dillapiol-PPh_3	70±5	84±5	95±3
Myristicin-PPh_3	75±3	91±2	97±3
Allyltetramethoxybenzene-PPh_3	83±4	88±3	98±1

Earlier we showed that the data of FTIR spectroscopy highly correlate with the data of the MTT test on cell survival [49,83]. The main cell structural units that contribute to the absorption of IR radiation can be distinguished (Figure 5): lipids of the cell membrane (2800-3000 cm^{-1}), proteins, especially transmembrane (1500-1700 cm^{-1}), phosphate groups of DNA (1240 cm^{-1}) and carbohydrates, including lipopolysaccharides (900–1100 cm^{-1}). Previously, we developed a technique for tracking the penetration and adsorption of the drug into cells using FTIR spectroscopy: dramatic changes in the intensity of the peaks of amide 1 and amide 2 indicate effective penetration of the drug into cells and vice versa [49,88,89].

Here we present the data of FTIR spectroscopy during online incubation of a suspension of HEK293T cells with apiol-PPh_3 in M-β-CD (Figure 5). Comparing the red spectrum (at 0 min incubation) and the black spectrum (after 60 min), it is obvious that there are practically no changes in the intensity of the peaks of amide I and II, characterizing the interaction of the drug with trans-membrane proteins, and indicating drug penetration. There is only a shift of the peak of amide 1 to the low-frequency region (inserts in Figure 5, the normalized intensity is shown), and amide 2 to the high-frequency with the simultaneous appearance of the shoulder. This indicates to the only adsorption of drug molecules on the cell surface, which is also confirmed by a weak increase in the intensity of the peaks at 2850-3000 cm^{-1} corresponding to the valence vibrations of the CH_2 groups (lipid bilayer). Thus, triphenylphosphine derivatives of allylbenzenes show only marginal activity against normal cells. For comparison, we present a positive control of the active and inactive reagent on the HEK293T cells (Figure 5b). Dramatic changes in the intensity of amide 1 and amide 2 peaks (Figure 5b, left) indicates to penetration of the model well cells membrane penetrating drug (doxorubicin) into the cells and effective cytostatic effect (according to MTT test). On the contrary, small changes in the intensity of the peaks of amide 1 and amide 2 indicates to weak penetration of the drug into the cells and non-cytostatic effect of doxorubicin in the composition with “intelligent” micelles (Figure 5b, right).

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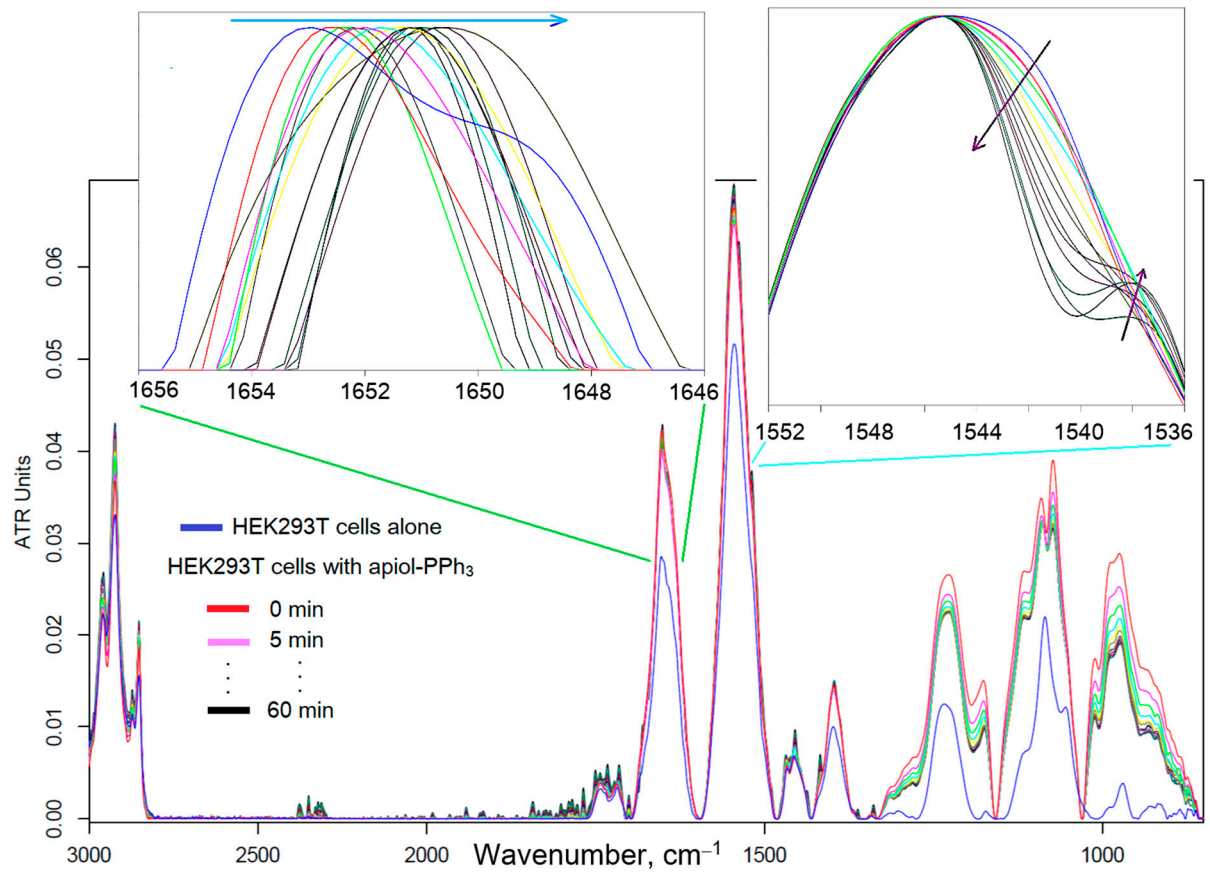
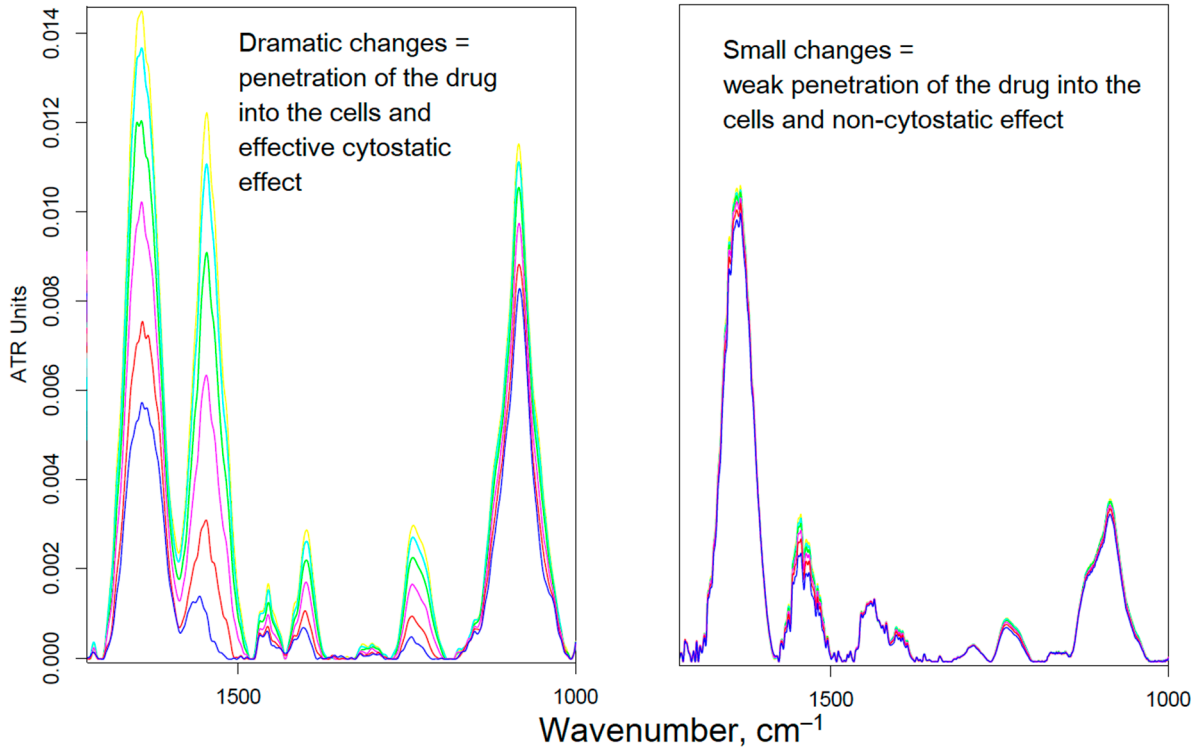


(a)



(b)

Figure 5. (a) FTIR spectra of HEK293T cells during online incubation (with step 5 min) with apiol-PPh_3 in the form of inclusion complexes with MCD. The inserts show enlarged fragments of peaks of amide I and II with a normalized intensity for better visualization of shifts of maxima. T = 37 °C. The

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inserts show enlarged fragments of peaks of amide I and II with a normalized intensity for better visualization of shifts of maxima. **(b)** FTIR spectra of HEK293T cells pre-incubated with doxorubicin (left), doxorubicin in "intelligent" micelles [88] (right) as a control of the correlation of changes in the intensity of peaks with the penetration and cytostatic effect of the drug.

3.5.2. Hemolytic Activity, Thrombogenicity and Phenotypic Sea Urchin Embryo Assay

Hemolytic activity and thrombogenicity are the primary parameter for evaluating the safety of medical formulations [90–92]. Phenotypic Sea Urchin Embryo Essay developed by colleagues is a visual way to study the toxicity of formulations *in vivo*. The sea urchin and human genomes contain more than 7000 common genes, including orthologs associated with a number of human diseases. From an evolutionary point of view, sea urchins are more closely related to humans than other model organisms. Therefore, they can be considered as a reliable and versatile model organism for studying the safety of new and existing cytotoxic formulations *in vivo*. Table 5 presents data on the % of erythrocyte hemolysis, the degree of whole blood thrombosis of apiol-PPh_3 and analogues, and data of phenotypic sea urchin embryo assay. Thus, the non-toxicity of PPh_3-derivatives of allylbenzenes for normal non-cancer cells, as well as the selectivity of action against cancer cells, is shown. The selectivity of cytotoxic action against cancer cells in comparison with normal cells can be explained by the fact that PPh3- cation provides selective accumulation and reduction of the mitochondrial membrane potential of the transformed cancer cells [93].

Table 5. Safety data on triphenylphosphine derivatives and paclitaxel.

Substance **X** in M-β-CD	Hemolysis index*, %	Thrombosis index**, %	Concentration causing changes in sea urchin embryos, μM
Paclitaxel	<0.5 (p = 0.012)	0.6±0.1	>4***
Apiol-PPh_3	0.8±0.2	1.1±0.2	
Dillapiol-PPh_3	0.9±0.2	1.0±0.1	
Myristicin-PPh_3	0.5±0.1	1.5±0.2	
Allyltetramethoxybenzene-PPh_3	0.7±0.1	0.7±0.2	

* For 0.1 mg/mL samples. The amount of released hemoglobin from erythrocytes relative to the control sample containing 0.05% Triton X-100. ** For 0.1 mg/mL samples. The amount of non-released hemoglobin in thrombus when H_2O was added relative to the control sample containing microscopic glass particles. *** $p<0.05$

4. Conclusion

In this paper, soluble forms (inclusion complexes in cyclodextrins or complexes with polyanionic polymer) of triphenylphosphine derivatives of allylbenzenes (individual components of plant (parsley) essential oils) are presented as potential independent cytostatic drugs (IC_{50} are in the micromolar concentration range (10^{-6} M) against A549) and as adjuvants to the classical cytotoxic drug paclitaxel. The positively charged PPh_3 fragment is used as an address label for the delivery of apiol and analogues in the mitochondria of cancer cells due to altered metabolism in cancer cells. Allylbenzene-PPh_3 enhances the effect of paclitaxel by 1.5-2 order in terms of cellular survival. At the same time, a high selectivity of the action of cytostatics against cancer cells is achieved, and the drugs practically do not act on healthy HEK293T model cells. In addition, the safety of triphenylphosphine formulations for erythrocytes, thrombosis and sea urchin embryos has been shown. In many cancers, mitochondrial membrane is more prone to depolarization, as compared to normal cells, which probably explains the observed selectivity of our compounds, since PPh3-derivatives are known to act as mitochondria-targeting agents. Further their efficacy as adjuvants may be most pronounced in combination (or in conjugation) with anticancer drugs whose mechanism of action affects mitochondria or mitochondrial membrane, which is an emerging field in cancer research.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, E.V.K., I.D.Z; methodology, I.D.Z., E.V.K., N.V.D., M.N.S., V.V.S.; formal analysis, I.D.Z.; investigation, I.D.Z, N.V.D., S.S.K.; data curation, I.D.Z.; writing—original draft preparation, I.D.Z.; writing—review and editing, E.V.K; project supervision, E.V.K.; funding acquisition, E.V.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Cell lines were obtained from Lomonosov Moscow State University Depository of Live Systems Collection (Moscow, Russia) HEK293T and A549 cells.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the main text and Supplementary Materials.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

γ-CD	γ-cyclodextrin
M-β-CD or MCD	methyl β-cyclodextrin

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