Fluorescein-Labeled Thiacalix[4]arenes as Potential Theranostic Molecules: Synthesis, Self-Association, and Antitumor Activity

Alan Akhmedov 1,*, Olga Terenteva 1, Evgenia Subakaeva 2, Pavel Zelenikhin 2, Ramilia Shurpik 1, Dmitriy Shurpik 1, Pavel Padnya 1 and Ivan Stoikov 1,3,*

Abstract: In this paper, a series of thiacalix[4]arenes were synthesized as potential theranostic molecules for antitumor therapy. We propose an original strategy for the regioselective functionalization of thiacalix[4]arene with a fluorescent label to obtain antiangiogenic agent mimetics. The aggregation properties of the synthesized compounds were determined using the dynamic light scattering. The average hydrodynamic diameter of self-associates formed by the macrocycles in 1,3-alternate conformation is larger (277–323 nm) than that of the similar macrocycle in cone conformation (185–262 nm). The cytotoxic action mechanism of the obtained compounds and their ability to penetrate into human lung adenocarcinoma and human duodenal adenocarcinoma cells were established using the MTT-test and flow cytometry. Thiacalix[4]arenes in 1,3-alternate conformation did not have a strong toxic effect. The toxicity of macrocycles in cone conformations on HuTu-80 and A549 cells (IC50 = 21.83–49.11 µg/mL) is shown. The resulting macrocycles are potential theranostic molecules that combine both the pharmacophore fragment for neoplasmas treatment and the fluorescent fragment for monitoring the delivery and biodistribution of nanomedicines.

Keywords: thiacalixarene; antitumor activity; theranostics; fluorescein; quaternary ammonium salts; A549; HuTu-80

1. Introduction

Cancer is a serious problem for modern society. In 2020, it is estimated that there were 19.3 million new cancer cases and nearly 10.0 million cancer deaths worldwide. New cases are forecast to reach 28.4 million in 2040, up 47% from 2020 [1]. Dysregulation of the immune response plays a significant role in the pathogenesis of cancer [2,3]. A number of mechanisms are known to allow tumor cells to form their microenvironment in order to suppress antitumor immunity [4,5]. One of these mechanisms is the tumor-associated production of galectins-1,3, which implement a wide range of extra- and intracellular functions [6–8]. Galectins-1,3 are involved in all stages of the tumor process [7]. Galectin-1 is a diagnostic marker of tumors [4], particularly tumors of the digestive tract (colon [9,10], liver [11,12], pancreas [13]), tumors of the respiratory system [14] and some lymphoid malignancies [15] and is also involved in angiogenesis and tumor growth [16].

In this regard, one of the modern types of anticancer drugs is galectin-1 inhibitors [17,18]. Increased drug resistance causes the overexpression of galectin-1 in malignant tumors; so-called multimodal therapy is used to combat this. Such multimodal therapy is an approach to cancer treatment that combines radiation and chemotherapy with several therapeutic methods [19]. Thus, multimodal therapy, including galectin-1 inhibitors, may increase the efficacy of co-administered drugs [17,18]. There are several different types of galectin-1
inhibitors, e.g., modified mono- and disaccharides containing galactose, or its analogs, non-carbohydrate-based inhibitors, such as peptides and peptidomimetics. The most successful peptide-based inhibitor of galectin-1 is anginex (βpep-25), a 33 amino acid peptide that exhibits antiangiogenic and antitumor effects [20].

Although anginex shows strong antitumor activity in vivo, non-peptide compounds are generally considered to be the preferred choice. Non-peptide compounds can potentially be administered orally without immune response and can also be optimized in terms of chemical and metabolic stability, resulting in better absorption and distribution to organs and tissues. Therefore, a series of topomimetics (calix[4]arene derivatives) based on anginex and partial peptidomimetics, taking into account the hydrophobic and hydrophilic fragments that are part of the anginex β-sheet, were synthesized [21]. Macrocycles PTX008 and PTX009 (Figure 1) have been identified as potent inhibitors of angiogenesis in cell proliferation and migration assays and in mouse models of ovarian cancer and melanoma [21,22]. This line of calixarene-based topomimetics has been patented by the Regents of the University of Minnesota as antibacterial, antiangiogenic, and antitumor agents, exhibiting the indicated activity in vitro and in vivo [23]. The mechanism of galectin-1 inhibition by calix[4]arene PTX008 (Figure 1) was studied by HSQC spectroscopy, and it was shown that PTX008 and anginex interact with galectin-1 through their hydrophobic and hydrophilic fragments [24]. In addition, a similar preparation based on the thiacalix[4]arene platform PTX014 was obtained (Figure 1), and its antitumor activity was shown [22].

![Figure 1. Calixarenes cytotoxic antitumor agents PTX008–PTX015.](image)

Modern macrocyclic systems have demonstrated unprecedented advantages in the diagnosis and therapy of neoplastic diseases in recent years, using the advantages of supramolecular chemistry [25–43]. Highly specific detection and topical therapy are still the main targets for theranostic anticancer agents. We proposed the idea of combining the properties of an anticancer drug and a diagnostic agent in one molecule to create theranostic molecules. As a pharmacophore fragment, it was proposed to use macrocycles analogs of anti-angiogenic agents PTX008–PTX015, in which one of the substituents of the fragments is covalently functionalized with fluorescein. In this work, we developed an original strategy for the regioselective functionalization of the thiacalix[4]arene platform to obtain fluorescein-containing analogs of PTX008–PTX015. This approach makes it possible to create theranostic molecules that combine both the pharmacophore fragment for the treatment of tumor neoplasms and the fluorescent fragment for monitoring the delivery and biodistribution of nanomedicines. The mechanism of the cytotoxic action of the obtained compounds and their ability to penetrate into cancer cells of human lung adenocarcinoma (A549) and human duodenal adenocarcinoma (HuTu-80) were determined by the MTT test and flow cytometry.

2. Materials and Methods

2.1. Chemistry

All reagents and solvents (Sigma-Aldrich, St. Louis, MO, USA) were used directly as purchased or purified according to the standard procedures. The $^1$H, $^{13}$C and $^1$H-$^1$H NOESY NMR spectra were recorded on an Avance 400 spectrometer (Bruker Corp., Billerica, MA, USA) (400 MHz for H-atoms) for 3–5% solutions in CDCl$_3$, DMSO-d$_6$. The residual solvent peaks were used as an internal standard. The FTIR ATR spectra were recorded
on the Spectrum 400 FT-IR spectrometer (Perkin Elmer, Seer Green, Lantrisant, UK) with a Diamond KRS-5 attenuated total internal reflectance attachment (resolution 0.5 cm⁻¹, accumulation of 64 scans, recording time 16 s in the wavelength range 400–4000 cm⁻¹). ESI HRMS experiments were performed at Agilent 6550 iFunnel Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA), equipped with Agilent 1290 Infinity II LC. Melting points were determined using the Boetius Block apparatus. Additional control of the purity of compounds and monitoring of the reaction were carried out by thin-layer chromatography using Silica G, 200 µm plates, UV 254.

2.2. Synthesis of Compounds 4a–b, 5a–d, 6a–d

Compounds 1 and 2 were synthesized according to published procedures [44–46]. Compounds 3a and 3b were synthesized according to published procedures. [45,47]

2.2.1. General Synthesis Procedure 4a–b

Compound 3a or 3b (0.5 g, 0.43 mmol) and N,N-dimethylpropane-1,3-diamine (52.10 mmol) were mixed in a round-bottom flask equipped with a magnetic stirrer. In the case of 3b, methanol (5 mL) was also added. The reaction mixture was stirred for 70 h at room temperature in the case of 3a. The reaction mixture was stirred for 24 h at room temperature, followed by 46 h under cooling in the case of 3b. Then, the solution was evaporated on a rotary evaporator, washed with water, and the residue was dried in a vacuum over phosphorus pentoxide. Products 4a–b were obtained as a white powder. Characterizations of synthesized compounds 4a–b are reported in the Supplementary Materials.

2.2.2. General Synthesis Procedure 5a–d

A solution of 0.35 g (0.294 mmol) of compound 4a (or 4b) in DMF was prepared in a round-bottom flask equipped with a magnetic stirrer. Then, a solution of 0.324 mmol of the compound (FITC or PhIC) in DMF was added to the flask. The reaction mixture was stirred for 24 h at room temperature. The solution was evaporated on a rotary evaporator until DMF was partially removed. Then, 75 mL of the corresponding ether (MTBE in the case of FITC, Et₂O in the case of PhIC) was added to the resulting concentrated solution. A precipitate formed; it was washed with the appropriate ether, and the residue was dried in vacuum over phosphorus pentoxide. Products 5a–d were obtained as bright orange or yellow powders. Characterizations of synthesized compounds 5a–d are reported in the Supplementary Materials.

2.2.3. General Synthesis Procedure 6a–d

A solution of 0.1 g (0.063 mmol) of compound 5a–d in methanol was prepared in a round-bottom flask equipped with a magnetic stirrer. Then 0.1 mL (32 mol) of methyl iodide was added to the flask. The reaction mixture was stirred for 24 h at room temperature. The solution was evaporated on a rotary evaporator, and the residue was dried in vacuum over phosphorus pentoxide. Products 6a–d were obtained as pale orange or yellow powders. Characterizations of synthesized compounds 6a–d are reported in the Supplementary Materials.

2.3. Determination of the Hydrodynamic Particle Size by Dynamic Light Scattering

The particle size was determined by the Zetasizer Nano ZS instrument (Worcestershire, UK) at 25 °C. The instrument contains a 4 mW He-Ne laser operating at a wavelength of 633 nm and incorporated noninvasive backscatter optics (NIBS). The measurements were performed at the detection angle of 173°, and the software automatically determined the measurement position within the quartz cuvette. Synthesized compounds 6a–d were dissolved completely in deionized water at concentrations used in the research (from 1 × 10⁻⁶ M to 1 × 10⁻³ M). Deionized water with resistivity >18.0 MΩ cm (Millipore-Q) was used for the preparation of the solutions.
2.4. Cytotoxicity of 6a–d on A549 and HuTu-80 Cell Lines

The ability of macrocyclic compounds to inhibit the viability and proliferative activity of A549 and HuTu-80 cells was determined using the MTT assay according to [48]. Briefly, cells were grown in 96-well plates in DMEM (GIBCO, Waltham, MA, USA) after supplementing with 10% FBS (Corning Inc., Corning, NY, USA), 100 units/mL penicillin (PanEco, Moscow, Russia) and 100 µg/mL streptomycin (PanEco, Russia), at 37 °C in a humidified atmosphere with 5% CO₂ up to 80% confluence. Then, the medium in wells was replaced with a fresh medium, supplemented with test substances in the concentration range of 0.5–100 µg/mL. The volume of the culture medium in the wells was 100 µL. After 24 h of cell incubation in the presence of agents, the medium in the wells was replaced with a fresh medium containing MTT (Merck) at a concentration of 0.5 mg/mL. Cells were incubated with MTT for 3 h (HuTu-80) or 4 h (A549) at 37 °C, then the medium from the wells was aspirated and 100 µL of dimethyl sulfoxide added. Probes were incubated at 37 °C for 15 min in the dark for the formazan crystals to dissolve. The optical density of the formazan solution in the wells was measured using a reader (BioRad xMark™ Microplate Spectrophotometer, Hercules, CA, USA) at a wavelength of 570 nm. Three series of experiments were carried out with at least 8 replications for each variant in the series.

2.5. Characterization of 6a–d Penetration into A549 and HuTu-80 Cells by Flow Cytometry

The macrocyclic compounds’ ability to penetrate into A549 and HuTu-80 cells was determined with a BD FACSCanto II flow cytometer. Cells were incubated for 2 h in the presence of test compounds at 37 °C and then stained with propidium iodide (PI), which selectively stains dead cells.

A549 and HuTu-80 cells were grown in DMEM (GIBCO, Waltham, MA, USA) after supplementing with 10% FBS (Corning, Inc., Corning, NY, USA), 100 units/mL penicillin (PanEco, Moscow, Russia) and 100 µg/mL streptomycin (PanEco, Moscow, Russia), at 37 °C in a humidified atmosphere with 5% CO₂. Cells were harvested and washed with fresh medium and then placed in individual sterile tubes at a concentration of 10⁵ cells/mL. After adding the test compounds to the tubes, the cell suspension was incubated for 2 h at 37 °C in the dark. Then the cell suspension was centrifuged at 2000 rpm for 5 min at room temperature, and cells were washed three times in phosphate-buffered saline (PBS, PanEco, Moscow, Russia). The cells were resuspended in 1 mL of PBS and transferred to cytometric tubes, when the samples were stained with 5 µL of PI solution (5 mg/mL), kept in the dark at room temperature for 2 min, and cytometric analysis was performed. The processing of cytometric data was carried out in the FACSDiva application.

3. Results and Discussion

3.1. Synthesis of Fluorescein- and Phenyl-Labeled thiacalix[4]arenes

To develop an approach to the design of macrocyclic drugs containing a covalently attached fluorescent label, it was proposed to synthesize various lower-rim substituted thiacalix[4]arenes containing tertiary amino groups and fluorescein fragment. Monophthalimide 2 (Scheme 1) was proposed as a precursor for the synthesis of target differently substituted p-tert-butythiacalix[4]arenes containing one fluorescent fragment [44]. At the first stage, compound 2 was synthesized according to the method of Ref. [44] (Scheme 1). It is known [44] that, regardless of the reaction conditions, the monosubstituted product 2 is formed in cone conformation, which opens up possibilities for further functionalization of the three unsubstituted hydroxyls. The formation of compound 2, apparently, is a consequence of two factors, i.e., the use of a bulky substituent (phthalimide group), which shields the phenolic groups of the macrocycle, and the formation of intramolecular hydrogen bonds between the carbonyl groups of the phthalimide fragment and the phenolic hydroxyl groups of thiacalix[4]arene. Intramolecular hydrogen bonds (OH···O=C) fix the phthalimide substituent in a position that prevents the next molecule of the alkylating agent from approaching the reaction center [44,45]. Next, the alkylation reaction of derivative 2 with ethyl bromoacetate was carried out. An analysis of the literature data showed that the
Pharmaceutics 2022, 14, 2340

5 of 12

alkylation of unsubstituted thiacalix[4]arene uses alkali metal carbonates as a template [49]. Thus, conformational stereoselectivity is easily controlled by selecting the appropriate alkali metal carbonate. In this case, the template effect is the main controlling factor. High selectivity is observed in acetone; the use of Na$_2$CO$_3$, K$_2$CO$_3$, and Cs$_2$CO$_3$ makes it possible to obtain cone, partial cone, and 1,3-alternate in 77, 58, and 78% yields, respectively [49]. The template effect in acetone can be explained by the fact that the intermediate phenolate precursors are more closely coordinated with the template metal ions [49]. Alkylation of compound 2 with ethyl bromoacetate in acetone at the boiling point of the solvent for 80 h using sodium or cesium carbonates made it possible to obtain compounds 3b [45] in cone conformation and 3a [45] in 1,3-alternate conformation, respectively (Scheme 1).

Further, the possibility of the aminolysis of the obtained compounds 3a–b with N,N-dimethylpropandiamine was studied. The use of heating led to an inversion of the conformations of the final products, and therefore, the reaction was carried out at room temperature for 70 h in methanol. Analysis of the $^1$H NMR spectra of the aminolysis products (Figures S1 and S2, ESI) showed no signals from the phthalimide group protons. The isolated aminolysis products are compounds in which the three ester groups of the starting compounds 3a–b reacted with N,N-dimethylpropandiamine to form amide groups, and the phthalimide group is absent. It was found that the reaction of compounds 3a–b with N,N-dimethylpropandiamine resulted in both methods, i.e., aminolysis of the ester groups and removal of the phthalimide protection with the formation of a primary amino group. The resulting compounds containing a primary amino group will be used for further functionalization of their various fragments, including fluorescein. Thus, thiacalix[4]arenes 4a–b were obtained in one step from triester derivatives 3a–b (cone and 1,3-alternate) in 80% and 88% yields, respectively (Scheme 1).

It is known [49] that the energy barrier to the inversion of the aryl fragment in thiacalix[4]arenes containing substituents less than four atoms long at the lower rim is low. In this regard, the conformation of compounds 4a–b containing a primary amino group was confirmed by the 2D $^1$H–$^1$H NOESY NMR spectroscopy. The $^1$H–$^1$H NOESY NMR spectrum of macrocycle 4a shows cross peaks between the protons of the tert-butyl groups and the methylene group bonded to the nitrogen atom as well as cross-peaks between the protons of the tert-butyl and amide groups, which indicates 1,3-alternate conformation of

![Scheme 1. Reagents and conditions: (i) N-(2-bromoethyl)phthalimide, Cs$_2$CO$_3$, (CH$_3$)$_2$CO; (ii) BrCH$_2$CO$_2$Et, Cs$_2$CO$_3$, (CH$_3$)$_2$CO; (iii) BrCH$_2$CO$_2$Et, Na$_2$CO$_3$, (CH$_3$)$_2$CO; (iv) NH$_2$(CH$_2$)$_3$N(CH$_3$)$_2$, CH$_3$OH; (v) NH$_2$(CH$_2$)$_3$N(CH$_3$)$_2$; (vi) FITC or PhIC, DMF; (vii) CH$_3$I, CH$_3$OH.](image-url)
The study of self-association of compounds 6a–b containing a fluorescein fragment was carried out in water in the concentration range from $1 \times 10^{-3}$ M to $1 \times 10^{-5}$ M. Synthesized compounds 6a–d were dissolved completely in deionized water at concentrations used in the research (from $1 \times 10^{-6}$ M to $1 \times 10^{-3}$ M). Deionized water with resistivity >18.0 MΩ cm (Millipore-Q) was used for the preparation of the solutions. The temperature of the solutions was maintained at 25 °C during the experiment. It was shown (Table 1) that the macrocycle 6a (1,3-alternate) form self-associates with a larger hydrodynamic diameter (285–587 nm) than the similar macrocycle 6b (cone) (199–353 nm). It should be noted that the solubility of compounds 6c–d containing the phenyl isocyanate fragment is lower than the water solubility of compounds 6a–b. The concentration range from $1 \times 10^{-4}$ M to $1 \times 10^{-6}$ M was used to study compounds 6c–d in water by the DLS method. The average
hydrodynamic diameter of self-associates formed by the macrocycle 6c (1,3-alternate) is larger (277–323 nm) than that of the similar macrocycle 6d (cone) (185–262 nm).

Thus, we can conclude that macrocycles 6a–d interact with cells as supramolecular self-associates.

3.3. Cytotoxicity of Synthesized Macrocycles

A series of experiments were performed using the A549 human lung adenocarcinoma cell line (which actively expresses galectin-1 [55]) and the HuTu-80 human duodenal adenocarcinoma cell line. The ability of 6a–d to inhibit the cells’ viability and proliferative activity was determined using the MTT test [48] after incubation for 24 h. It was found that 6a and 6c (1,3-alternate) did not reduce the viability of A549 cells over the entire range of concentrations studied (0.5–100 µg/mL) (Figure 2). It was also shown that 6b and 6d (cone) had a cytotoxic effect on A549 cells at concentrations of ≥50 µg/mL (6b) and ≥25 µg/mL (6d) (Figure 2).

![Figure 2. Cytotoxicity of compounds 6a–d on cell line A549.](image)

Experiments with the HuTu-80 cell line showed that compound 6a (1,3-alternate) did not have the ability to reduce the viability of HuTu-80 cells at a concentration of ≤50 µg/mL (Figure 3). The cytotoxic activity of compound 6a was fixed at the concentration of 100 µg/mL, and the viability of HuTu-80 cells after treatment with 6a was 0.67 ± 0.05. The macrocycles 6b (cone) and 6c (1,3-alternate) were cytotoxic to HuTu-80 cells at concentrations ≥ 25 µg/mL (Figure 3). The cytotoxic effect of 6d (cone) was negligible at concentrations ≤4 µg/mL (Figure 3). Exposure to compound 6d increased with the increasing concentration and was significant at concentrations 50 µg/mL or more. At concentrations above 50 µg/mL, 6d almost completely eliminated HuTu-80 cells.

The results of the analysis of the cytotoxic activity of macrocyclic compounds in relation to HuTu-80 cells correspond to the data obtained in relation to A549 cells. It was shown that the cell line HuTu-80 was more sensitive to the effects of these substances. For all studied samples of macrocyclic compounds, the average inhibitory concentration (IC₅₀) was calculated (Table 2). Summarizing the presented data, thiacalix[4]arenes 6a and 6c (1,3-alternate) did not have a strong toxic effect. The IC₅₀ value of substance 6b (cone) for HuTu-80 cells was 49.11 µg/mL. Compound 6d (cone) was shown to have the highest toxic properties, with IC₅₀ 21.83 µg/mL and 37.55 µg/mL for HuTu-80 and A549 cells, correspondingly (Table 2). Thus, the conformation of trisubstituted macrocycles affects their cytotoxicity; as a rule, compounds in cone conformation are more toxic than macrocycles in 1,3-alternate conformation.
We can conclude that FITC-containing compound 6b (cone) has a lower toxic effect compared to the similar PhIC-containing compound 6d (cone) both on A549 and HuTu-80 cells (Table 2). Apparently, the size of the macrocycle affects the efficiency of the interaction of the compound with the cell. If we compare the toxic effect of compounds 6a–d with similar compounds based on tetrasubstituted macrocycles (in cone conformations) PTX008–PTX015 (Table 2), we can see that the trisubstituted compounds have a lower toxic effect. Apparently, this is due to the inhibition of galectin, which is carried out by terminal amino groups. Thus, four fragments of tetrasubstituted calixarenes inhibit galectin more efficiently, unlike three fragments in compounds 6b and 6d. This assumption correlates with previously published data on the putative mechanism of the cytotoxic effect of PTX008–PTX015 [22].

Thus, the antiproliferative and cytotoxic activity of synthesized compounds 6a–d as analogs of the anti-angiogenic agents were evaluated. It was found that macrocycles 6b,d (cone) are more cytotoxic than macrocycles 6a,c (1,3-alternate). However, the cytotoxicity of the obtained compounds is lower than similar PTX008–PTX015 compounds, which is explained by the smaller number of terminal amino groups and correlates with the proposed mechanism of action of PTX008–PTX015 compounds [22].

### 3.4. Penetration into A549 and HuTu-80 Cells of Synthesized Macrocycles

The next step of the work was to determine the ability of macrocyclic compounds 6a–d to penetrate into A549 and HuTu-80 cells using flow cytometry with propidium iodide co-staining. It was found that associates of compounds 6a–d after 2 h of incubation penetrate into both living and dead A549 and HuTu-80 cells (Figure 4), and the penetrating ability of

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Table 2. IC$_{50}$ values of the macrocycles.

| Compounds       | $IC_{50}$, µg/mL |
|-----------------|------------------|
|                 | HuTu-80          | A549             |
| 6a (1,3-alternate) | >100$^a$         | >100$^a$         |
| 6b (cone)       | 49.11            | >100$^a$         |
| 6c (1,3-alternate) | >100$^a$         | >100$^a$         |
| 6d (cone)       | 21.83            | 37.55            |
| PTX008 (cone)   | nd               | 1.87$^b$         |
| PTX013 (cone)   | nd               | 0.87$^b$         |
| PTX014 (cone)   | nd               | 8.28$^b$         |

$^a$ IC$_{50}$ have not been reached in the studied concentration range. $^b$ Data from literary sources [22] “nd” indicates no data.
Thiacalix[4]arenes is quite high. It should be noted that 6a–d, when incubated with cells, had a toxic effect on both A549 and HuTu-80 cells. The proportion of dead cells in the presence of 6a–d increased significantly. The cytotoxicity of the studied compounds 6a–d depended on the concentration and reached the highest values for agent concentrations of 100 µg/mL. In this study, thiacalix[4]arenes can be ranked according to the cytotoxicity exerted on A549 cells in the order 6a → 6c → 6b → 6d. It can be noted that the macrocycles 6b and 6d (cone) had a higher toxic effect on A549 cells than macrocycles 6a and 6c (1,3-alternate). Compound 6a had the worst penetrating ability, staining only 36.6% and 61% of A549 cells at concentrations of 5 µg/mL and 10 µg/mL, respectively. On the HuTu-80 cell line, thiacalix[4]arenes can be ranked according to increasing cytotoxicity in the order 6c → 6a → 6b → 6d. It can be noted that both in the case of the A549 cell line and in the case of the HuTu-80 cell line, macrocycles 6b and 6d (cone) had a higher toxic effect on cells than macrocycles 6a and 6c (1,3-alternate). At concentrations of 5 µg/mL and 10 µg/mL, all tested compounds stained only up to 50% of the HuTu-80 cells.

**Figure 4.** Penetration of thiacalix[4]arenes 6a–d into A549 (a) and HuTu-80 (b) cells.

The ability of macrocycles 6a–d to penetrate into A549 and HuTu-80 cell lines was evaluated. In conclusion, one can say that macrocycles penetrate into living and dead cells; the cytometric cytotoxic profiles confirm the MTT test data.

4. Conclusions

Thus, an approach to create potential theranostic molecules with both a pharmacophore fragment and a fluorescent fragment was proposed and implemented. Phenylisocyanate- and fluorescein-containing analogs of antiangiogenic agents PTX008–PTX015 were obtained by the original regioselective method of the functionalization of thiacalix[4]arene. All obtained compounds were characterized by 1H, 13C NMR, IR spectroscopy, and HRMS. Using the DLS method, it was established that the synthesized macrocycles form self-associates only in aqueous solutions with average hydrodynamic diameters of 166–465 nm. The antiproliferative and cytotoxic activity of the synthesized compounds 6a–d (analogs of the anti-angiogenic agents PTX008–PTX015) was evaluated by the MTT test and confirmed cytometrically. It was found that macrocycles 6b,d (cone) are more cytotoxic than macrocycles 6a,c (1,3-alternate). It was also shown that macrocycles can penetrate both living and dead A549 and HuTu-80 cancer cells. The resulting macrocycles are potential theranostic molecules that combine both the pharmacophore fragment for the treatment of tumor neoplasms and the fluorescent fragment for monitoring the delivery and biodistribution of nanomedicines.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pharmaceutics14112340/s1, Characterization of compounds 4a-b, 5a-d, 6a-d; Figures S1–S10. 1H NMR spectra of compounds 4a-b, 5a-d, 6a-d; Figures S11–S20. 13C NMR spectra of compounds 4a-b, 5a-d, 6a-d; Figures S21–S30. FT-IR spectra of compounds 4a-b, 5a-d, 6a-d; Figures S31–S40. HRMS spectra of compounds 4a-b, 5a-d, 6a-d; Figures S41 and S42. 1H-1H NOESY spectra of compounds 4a-b.

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