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SHORT COMMUNICATION

Search for human DNA topoisomerase II poisons in the group of 2,5-disubstituted-1,3,4-thiadiazoles

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Abstract

A series of six 2,5-disubstituted 1,3,4-thiadiazole derivatives was synthesized and examined for cytotoxic activity in MCF-7 and MDA-MB-231 breast cancer cells. MTT assay confirmed that 2-(3-fluorophenylamino)-5-(3-hydroxyphenyl)-1,3,4-thiadiazole (**2**), 2-(4-bromophenylamino)-5-(2,4-dichlorophenyl)-1,3,4-thiadiazole (**3**), 2-(4-fluorophenylamino)-5-(2,4-dichlorophenyl)-1,3,4-thiadiazole (**4**), had ability to inhibit MCF-7 and MDA-MB-231 cells proliferation. The IC_{50} values for the mentioned compounds ranged between 120 and 160 μM (with respect to MCF-7 cells) and from 70 to 170 μM (with respect to MDA-MB-231 cells). It turned out, moreover, that compound **2** is a human topoisomerase II (topoII) catalytic inhibitor whereas the two other compounds (i.e. **3** and **4**) are capable of stabilizing DNA-topoII cleavage complex and thus are topoII poisons.

Keywords

1,3,4-Thiadiazoles, breast cancer, cytotoxicity, MTT assay, topoisomerase inhibitors

History

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Introduction

Cancer is one of the greatest challenges of contemporary medicine. It affects people living in all regions of the world and belonging to all social groups. Currently, the mortality resulting from cancer exceeds the number of deaths caused by HIV/AIDS, tuberculosis and malaria altogether. It is estimated that in 2012, approximately 14 million new cases of cancer were diagnosed and approximately 8 million deaths resulting from this disease were recorded worldwide. Due to increasing Earth's population and a longer life expectancy, it is predicted that as soon as in 2030 there will be 22 million new cases of cancer and 13 million deaths[1]. Attempts to counteract this alarming tendency involve, among other things, designing new methods that enable early diagnosis and introducing new anticancer drugs to clinical practice[2]. 1,3,4-Thiadiazoles are a group of compounds known to have potential effect on cancer cells[3,4]. Their anticancer activity may be achieved via different molecular mechanisms, including cyclin-dependent kinase (CDK) inhibition[5], Abl tyrosine-kinase inactivation[6], down-regulation of Bcl-X_L expression and inhibition of Akt/PKB[7], inhibition of the so-called tumor-associated carbonic anhydrase isozymes I, II, IX[8]. To date, a little is known about the ability of 1,3,4-thiadiazole derivatives to act against cancer cells through the topoisomerase-associated mechanism[9]. However, it was demonstrated that certain 1,3,4-thiadiazoles inhibit DNA biosynthesis[10], which, in turn, may be associated with their potential anti-topoisomerase activity.

Topoisomerases belong to the family of enzymes that catalyze changes in the spatial structure of DNA. They enable conformational changes in DNA topology necessary for transcription, replication and recombination of genetic material[11]. Thanks to them, amplification of genetic material is possible, which is subsequently delivered to daughter cells formed by cell division. Due to inhibiting topoisomerases activity, DNA synthesis in cells is limited and consequently cell division is reduced. Substances targeting topoisomerases fall into two categories – topoisomerase poisons and topoisomerase catalytic inhibitors[12]. The term ''topoisomerase poisons'' reflects the fact that these compounds convert this enzyme into a cell poison, which causes a range of irreversible damage to genetic material[13]. The effect of topoisomerase poisons depends on the concentration of this enzyme in cells. The level of topoisomerases is the highest in rapidly proliferating cells, which include cancerous cells[14]. Therefore, topoisomerase poisons, and especially topoisomerase II (topoII) poisons are known from their anticancer properties. It is also known that topoisomerase poisons exhibit considerably higher cytotoxicity than topoisomerase catalytic inhibitors[13]. This is why our current research focuses on searching for compounds that inhibit human DNA topoII activity by stabilizing the cleavable complex. We assumed that the probability of finding compounds with desirable anticancer properties is high in the group of 1,3,4-thiadiazole derivatives. Particularly 2,5-disubstituted 1,3,4-thiadiazoles demonstrated the ability to inhibit cancer

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cell proliferation and exhibited weaker influence on the viability of normal cells[15–17]. Inspired by these observations, we designed and synthesized a group of 1,3,4-thiadiazole derivatives differing in the structure of the substituents at C2 and C5 position. Mono- and disubstituted aryl rings, containing electron-donating and electron-withdrawing groups, were chosen to test the influence of the substitution pattern and electronic properties of the substituents on anticancer activity of 1,3,4-thiadiazole derivatives. The obtained compounds subsequently underwent biological tests with the use of MCF-7, MDA-MB-231 breast cancer cells and human normal skin fibroblasts. The mentioned cancer cell lines were chosen for our studies as it had been demonstrated that compounds inhibiting topoII were frequently effective against these breast cancer cells[18,19]. Finally, the most potent compounds were also investigated for their ability to act as poisons or catalytic inhibitors of human DNA topoII.

Materials and methods

Chemistry

All reagents were purchased from Alfa-Aesar and Sigma-Aldrich, and used without further purification. Melting points were determined by using Fischer-Johns apparatus (Sanyo, Osaka, Japan) and are uncorrected. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance instrument using DMSO-d_6 as a solvent and TMS as an internal standard. Chemical shifts are expressed as δ (ppm). The purity of the compounds was checked by TLC on plates precoated with silica gel Si 60 F_{254}, produced by Merck Co. (Darmstadt, Germany). The spots were detected by exposure to UV-lamp at $\lambda = 254$ nm. Elemental analyses were performed on AMZ 851 CHX analyzer and the results were within ±0.4% of the theoretical value.

*Synthesis of 1,3,4-thiadiazole derivatives (**1–6**)*

A solution of 0.01 mol of respective aryl hydrazide (3-chlorobenzhydrazide for **1**, 3-hydroxybenzhydrazide for **2**, 2,4-dichlorobenzhydrazide for **3** and **4**, 2,4-dichlorophenoxyacetic acid hydrazide for **5** and **6**) and equimolar amounts of appropriate aryl isothiocyanate (3-fluorophenyl isothiocyanate for **1** and **2**, 4-bromophenyl isothiocyanate for **3** and **5**, 4-fluorophenyl isothiocyanate for **4** and **6**) in 25 ml of anhydrous EtOH was heated under reflux for 5 min. Next, the solution was cooled and the solid formed was filtered off, washed with diethyl ether and crystallized from EtOH. Subsequently, thus obtained thiosemicarbazide derivatives were dissolved in concentrated sulfuric acid (15 ml) and stirred at room temperature for 2 h. Then, the reaction mixture was poured onto crushed ice and the solid precipitated was filtered off, washed several times with water, dried and crystallized from EtOH to give products **1–6**.

*2-(3-Fluorophenylamino)-5-(3-chlorophenyl)-1,3,4-thiadiazole (**1**).* Yield 75%, m.p. 210–212 °C. ^{1}H-NMR (300 MHz) (DMSO-d_6) δ (ppm): 6.88 (td, 1H, ArH, $J = 2.5$ Hz, 8.5 Hz), 7.25 (dd, 2H, ArH, $J = 2.0$ Hz, 8.5 Hz), 7.51–7.95 (m, 4H, ArH), 8.11 (dd, 1H, ArH, $J = 2.5$ Hz, 11.8 Hz), 10.87 (s, 1H, NH, exchangeable with D_2O). ^{13}C-NMR (75 MHz) (DMSO-d_6) δ (ppm): 112.20, 126.15, 126.54, 129.30, 130.05, 130.64, 131.13, 131.73, 132.30, 132.51, 134.43, 142.66, 157.32, 164.63. Anal. Calc. for $C_{14}H_9ClFN_3S$ (305.76): C 54.99, H 2.97, N 13.74. Found: C 55.08, H 3.10, N 13.70.

*2-(3-Fluorophenylamino)-5-(3-hydroxyphenyl)-1,3,4-thiadiazole (**2**).* Yield 69%, m.p. 136–138 °C. ^{1}H-NMR (300 MHz) (DMSO-d_6) δ (ppm): 6.75–8.18 (m, 8H, ArH), 10.77 (s, 1H, NH, exchangeable with D_2O), 11.25 (s, 1H, OH, exchangeable with D_2O). ^{13}C-NMR (75 MHz) (DMSO-d_6) δ (ppm): 113.47, 113.89, 114.82, 117.80, 118.08, 118.41, 128.66, 130.95, 131.70, 132.81, 142.56, 154.26, 158.36, 158.83, 161.49, 164.00, 164.61. Anal. Calc. for $C_{14}H_{10}FN_3OS$ (287.31): C 58.53, H 3.51, N 14.63. Found: C 58.40, H 3.69, N 14.45.

*2-(4-Bromophenylamino)-5-(2,4-dichlorophenyl)-1,3,4-thiadiazole (**3**).* Yield 80%, m.p. 250–252 °C. ^{1}H-NMR (300 MHz) (DMSO-d_6) δ (ppm): 7.53 (d, 2H, ArH, $J = 8.8$ Hz), 7.60 (dd, 1H, ArH, $J = 2.1$ Hz, 8.6 Hz), 7.65 (d, 2H, ArH, $J = 8.8$ Hz), 7.84 (d, 1H, ArH, $J = 2.0$ Hz), 8.10 (d, 1H, ArH, $J = 8.3$ Hz), 10.74 (s, 1H, NH, exchangeable with D_2O). ^{13}C-NMR (75 MHz) (DMSO-d_6) δ (ppm): 114.00, 120.04, 128.41, 128.61, 130.50, 132.07, 132.15, 132.35, 135.82, 140.09, 152.90, 165.92. Anal. Calc. for $C_{14}H_8BrCl_2N_3S$ (401.11): C 41.92, H 2.01, N 10.48. Found: C 41.76, H 1.90, N 10.55.

*2-(4-Fluorophenylamino)-5-(2,4-dichlorophenyl)-1,3,4-thiadiazole (**4**).* Yield 76%, m.p. 228–230 °C. ^{1}H-NMR (300 MHz) (DMSO-d_6) δ (ppm): 7.10–7.26 (m, 2H, ArH), 7.60 (dd, 1H, ArH, $J = 2.2$ Hz, 8.6 Hz), 7.65–7.73 (m, 2H, ArH), 7.84 (d, 1H, ArH, $J = 2.1$ Hz), 8.11 (d, 1H, ArH, $J = 8.5$ Hz), 10.63 (s, 1H, NH, exchangeable with D_2O). ^{13}C-NMR (75 MHz) (DMSO-d_6) δ (ppm): 116.04, 116.34, 119.82, 119.94, 128.49, 128.59, 130.46, 132.03, 132.08, 135.72, 137.30, 152.41, 156.33, 159.50, 166.43. Anal. Calc. for $C_{14}H_8Cl_2FN_3S$ (340.20): C 49.43, H 2.37, N 12.35. Found: C 49.65, H 2.23, N 12.38.

*2-(4-Bromophenylamino)-5-[(2,4-dichlorophenoxy)methyl]-1,3,4-thiadiazole (**5**).* Yield 84%, m.p. 170–172 °C. ^{1}H-NMR (300 MHz) (DMSO-d_6) δ (ppm): 5.53 (s, 2H, CH_2), 7.36 (d, 1H, ArH, $J = 9.0$ Hz), 7.42 (dd, 1H, ArH, $J = 2.4$ Hz, 8.9 Hz), 7.51 (d, 2H, ArH, $J = 8.9$ Hz), 7.57–7.64 (m, 3H, ArH), 10.52 (s, 1H, NH, exchangeable with D_2O). ^{13}C-NMR (75 MHz) (DMSO-d_6) δ (ppm): 65.90, 113.82, 116.58, 119.90, 123.23, 126.11, 128.67, 129.95, 132.27, 140.15, 152.40, 155.88, 165.69. Anal. Calc. for $C_{15}H_{10}BrCl_2N_3OS$ (431.13): C 41.79, H 2.34, N 9.75. Found: C 41.67, H 2.18, N 9.85.

*2-(4-Fluorophenylamino)-5-[(2,4-dichlorophenoxy)methyl]-1,3,4-thiadiazole (**6**).* Yield 80%, m.p. 184-185°C. ^{1}H-NMR (300 MHz) (DMSO-d_6) δ (ppm):5.52 (s, 2H, CH_2), 7.15–7.23 (m, 2H, ArH), 7.34–7.44 (m, 2H, ArH), 7.60–7.68 (m, 3H, ArH), 10.49 (s, 1H, NH, exchangeable with D_2O). ^{13}C-NMR (75 MHz) (DMSO-d_6) δ (ppm): 65.84, 115.97, 116.27, 116.59, 119.66, 119.77, 123.27, 126.10, 128.64, 129.94, 137.38, 152.42, 155.39, 166.15. Anal. Calc. for $C_{15}H_{10}Cl_2FN_3OS$ (370.23): C 48.66, H 2.72, N 11.35. Found: C 48.68, H 2.60, N 11.23.

Computational studies

Automated docking setup

Docking studies were conducted by means of the FlexX program[20] as implemented in LeadIT package (BioSolveIT, Sankt Augustin, Germany)[21] using a model of human DNA topoII binding site complexed with etoposide (PDB id: 3QX3[22]) as a native ligand. The ligand within the active site and all water molecules were removed, while magnesium ion was allowed to remain with the charge of +2. The active site was defined to include all atoms within 6.5 Å radius of the native ligand. The first 100 top ranked docking poses were saved for each docking run. To validate the molecular docking protocol, etoposide was initially docked into the crystal structure of the enzyme. The docked ligand was found to have similar binding pose to the co-crystallized molecule. Subsequently, compounds **1–6** were docked using same docking parameters.

Semiempirical and DFT calculations

Conformational search was calculated using HyperChem 8.0.1 (Hypercube, Gainesville, FL)[23]. Structures were optimized to the closest local minimum at the semiempirical level using RM1 parametrization. Convergence criterion was set to $0.01\,kcal\,mol^{-1}\,Å^{-1}$. Electrostatic potentials were calculated using the B3LYP functional[24,25] expressed in the basis set of 6–31 G(d)[26–28] as implemented in Gaussian 09 (Gaussian Inc., Wallingford, CT)[29]. Natural population analysis (NPA) phase of NBO was used[30]. The highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals, as well as electrostatic potentials maps were illustrated using the GaussView 5.0 program (Gaussian Inc., Wallingford, CT)[31].

Biological assays

Materials

Stock cultures of human MCF-7 and MDA-MB-231 breast cancer cells as well as normal human skin fibroblasts were purchased from the American Type Culture Collection (Manassas, VA). Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in a cell culture were products of Gibco (Waltham, MA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals, Inc. (Gaithersburg, MD). [^{3}H]thymidine (6.7 Ci/mmol) was purchased from NEN (Boston, MA) and Scintillation Cocktail ''Ultima Gold XR'' from Packard (Downers Grove, IL). Sodium dodecyl sulfate (SDS) was received from Bio-Rad Laboratories (Hercules, CA). Etoposide and topoII drug screening kit were purchased from TopoGEN (Columbus, OH). MTT and serum replacement I (CPSR1) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture

Human MDA-MB-231 and MCF-7 breast cancer cells maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin at 37 °C. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin and 0.02% EDTA in calcium-free phosphate buffered saline (PBS), counted in hemocytometers and plated at 5×10^5 cells/well of six-well plates (Nunc) in 2 ml of growth medium (DMEM without phenol red with 10% CPSR1). Cells reached about 80% of confluency at day 3 and in most cases such cells were used for the assays.

Cell viability assay

The assay was performed according to the method followed by Carmichael et al.[32], using MTT. Confluent cells, cultured for 24 h with various concentrations of the studied compounds in six-well plates were washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37 °C in 5% CO_2 in an incubator. The medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added to the cells attached. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability of breast cancer cells and normal human skin fibroblasts cultured in the presence of compounds **1–6** was calculated as percentage of control cells.

[^{3}H]thymidine incorporation assay

To examine the effect of the studied compounds on cells proliferation, MCF-7 and MDA-MB-231 cells, as well as normal human skin fibroblasts were seeded in six-well tissue culture dishes at 1×10^5 cells/well with 1 ml of growth medium. After 48 h ($1.8 \pm 0.1 \times 10^5$ cells/well), plates were incubated with varying concentrations of compounds **2–4**, etoposide and 0.5 μCi of [^{3}H]thymidine for 24 h at 37 °C. Cells were rinsed three times with PBS, solubilized with 1 ml of 0.1 M sodium hydroxide containing 1% SDS, scintillation fluid (9 ml) was added and radioactivity was determined by liquid scintillation counting. [^{3}H]thymidine uptake was expressed as dpm/well.

Relaxation assay of topoII

Supercoiled pRYG DNA (0.5 μg) was incubated with 4U of human topoII in the cleavage buffer [30 mM Tris–HCl (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 3 mM ATP, 15 mM mercaptoethanol], in the presence of varying concentrations of the studied compounds. Reactions were carried out at 37 °C for 1 h and then terminated by the addition of 2 μl, 10% SDS and 2 μl, 50 μg/ml proteinase K. The reaction mixture was subjected to electrophoresis through a 0.8% agarose gel containing 0.5 mg/ml ethidium bromide in TBE buffer (90 mM Tris–borate and 2 mM EDTA). The gels were stained with ethidium bromide and photographed under UV light.

Results

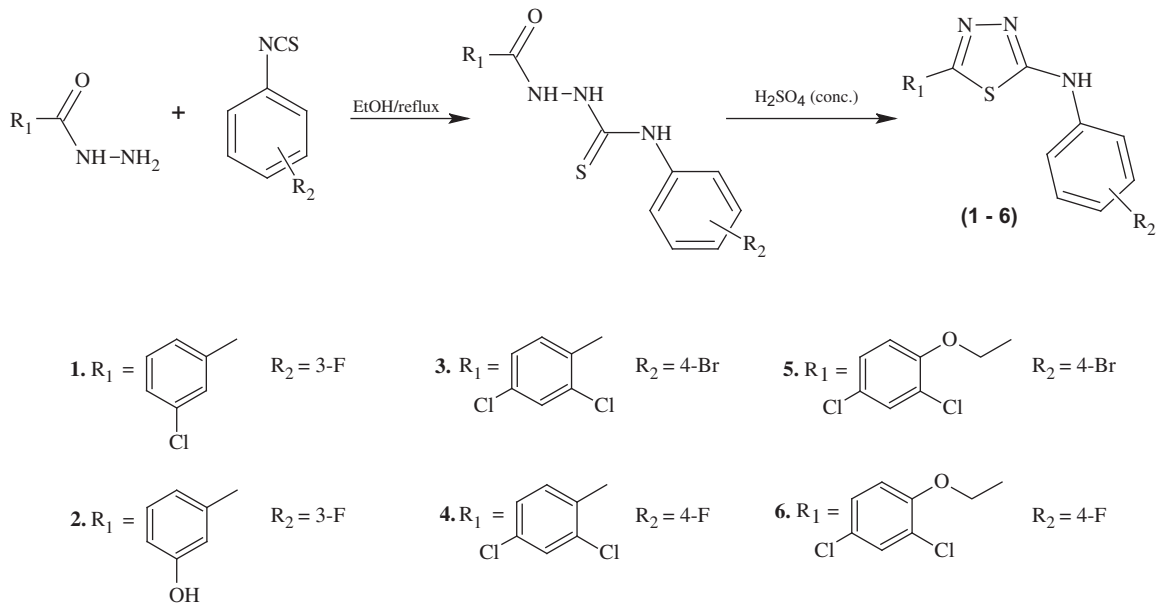
In the process of designing potential DNA topoII poisons, a molecular docking method was used to explore interactions between 1,3,4-thiadiazole derivatives and DNA-binding pocket of human DNA topoII. Since the ability of simultaneous interaction with a DNA strand and enzyme is an inherent feature of compounds stabilizing the topoII-DNA cleavable complex, the action of topoII poisons must occur within the DNA-dependent subunit of topoII. The model of this subunit, obtained from Protein Data Bank (id: 3QX3), was used in docking simulations. It was found that compounds **1–6** show a strong affinity to DNA-dependent subunit of topoII and interact both with the enzyme and nucleobases of the DNA (Figure S1).

In the majority of compounds, the docking scores were comparable or even superior to etoposide – an anticancer drug which is a well-known topoII poison (Table 1). Therefore, the designed 2,5-disubstituted 1,3,4-thiadiazole derivatives were synthesized in a two-stage reaction (Scheme 1), and subsequently subjected for biological testing on normal and cancer cell lines. As had been demonstrated before, compounds inhibiting topoII were frequently effective against breast cancer cells[18,19]. Bearing this is mind, the effect of compounds **1–6** on the viability of estrogen receptor-positive (MCF-7) and estrogen receptor-negative (MDA-MB-231) breast cancer cells was evaluated. Moreover, etoposide was used as a reference drug ($IC_{50} = 125 \pm 2$μM in MCF-7 and 98 ± 2μM in MDA-MB-231 cells). Viability of breast

Table 1. Docking scores of compounds **1–6** and etoposide obtained for DNA-dependent subunit of human topoII.

	Compounds						
	1	**2**	**3**	**4**	**5**	**6**	Etoposide
DNA-binding site (PDB: 3QX3)	−20.59	−23.69	−19.42	−20.74	−15.40	−20.35	−20.61

Energy units are in kcal/mol.



Scheme 1. Synthetic route to compounds **1–6**.

Table 2. IC_{50} values (μM) of compounds **1–6** and etoposide in breast cancer cells (MCF-7 and MDA-MB-231) and normal human skin fibroblasts using MTT assay.

	Compounds						
Cells	**1**	**2**	**3**	**4**	**5**	**6**	Etoposide
MCF-7	>200	140	120	160	>200	>200	125
MDA-MB-231	>200	170	70	130	>200	>200	98
Fibroblasts	>200	>200	>200	170	200	>200	55

The error for compounds **1–6** and etoposide is ±2 μM. Mean values ± SD from three independent experiments done in duplicate are presented.

cancer cells was measured by the method of Carmichael et al.[32] using tetrazolium salt (MTT).

The MTT assay showed that compounds **2**, **3** and **4** were endowed with an ability to inhibit MCF-7 and MDA-MB-231 cells proliferation (Table 2). The IC_{50} values for compounds **2–4** ranged between 120 and 160 μM (with respect to MCF-7 cells) and from 70 to 170 μM (with respect to MDA-MB-231 cells). Among the active derivatives, 2-(4-bromophenylamino)-5-(2,4-dichlorophenyl)-1,3,4-thiadiazole (**3**) proved to be more potent than etoposide with respect to both types of cancer cells (with IC_{50} values in MCF-7 of 120 ± 2 μM, and in MDA-MB-231 of 70 ± 2 μM). The assessment of viability of human normal skin fibroblasts exposed to the active compounds (i.e. derivatives **2–4**) demonstrated that they are characterized with a considerably lower toxicity than etoposide (IC_{50} > 200 μM for **2** and **3** versus 55 ± 2 μM for etoposide) and exhibit greater specificity to breast cancer cells (MCF-7 and MDA-MB-231). In order to check whether the loss of viability of breast cancer cells (MCF-7 and MDA-MB-231) results from reduced proliferation, we analyzed the influence of compounds **2–4** on the process of DNA biosynthesis (Table 3).

All the three 1,3,4-thiadiazole derivatives inhibited intracellular DNA synthesis, but in MCF-7 cells compound **2** turned out to be the most potent (IC_{50} = 80 ± 2 μM), and in MDA-MB-231 cells – compound **3** (IC_{50} = 90 ± 2 μM). The derivatives of 1,3,4-thiadiazole, in contrast to etoposide, demonstrated higher selectivity as far as DNA biosynthesis in cancer cells and fibroblasts was concerned (e.g. IC_{50} for **2** was 80 ± 2 μM and >200 μM, and for etoposide 75 ± 2 μM and 95 ± 2 μM towards MCF-7 and fibroblasts, respectively). One of the causes of inhibiting DNA biosynthesis by compounds **2–4** may be their influence on enzymes that take part in the process of genetic material replication. One of the most essential enzymes required for such a process are topoisomerases. The effect of the active compounds (**2–4**) on the activity of human DNA topoII was tested with the use of relaxation assay kit for topoII. This type of kit, i.e. plasmid-based screening kit, enable not only identification of compounds inhibiting topoII, but also distinguishes topoII catalytic inhibitors from topoII poisons. Intensity of the electrophoretic band

Table 3. Cytotoxic effects of compounds **2–4** and etoposide on the cultured breast cancer cells (MCF-7 and MDA-MB-231) and normal human skin fibroblasts as measured by inhibition of [^{3}H]thymidine incorporation into DNA.

	Compounds			
Cells	**2**	**3**	**4**	Etoposide
MCF-7	80	95	135	75
MDA-MB-231	170	90	140	72
Fibroblasts	>200	>200	160	95

The error for compounds **2–4** and etoposide is ±2 μM. Mean values ± SD from three independent experiments done in duplicate are presented.

corresponding to linear DNA (pHOT1) is increased when the tested compound acts as a topoII poison. On the other hand, in case of topoII catalytic inhibitors there is a clearly visible loss of decatenated kDNA products[33].

The previously conducted studies showed that the concentration of etoposide required for 50% reduction in topoII activity was found to be 100 μM[19]. The current tests reveal that a total inhibition of topoII enzymatic activity occurs at a concentration of 200 μM. Worth mentioning is the fact that 1,3,4-thiadiazole derivatives **2**–**4** completely inhibited the activity of this enzyme at a concentration lower by a half (i.e. at 100 μM). Furthermore, it turned out that compound **2** is a topoII catalytic inhibitor whereas the two other compounds (i.e. **3** and **4**) are capable of stabilizing DNA–topoII cleavage complex and thus are topoII poisons.

Discussion

Of the 1,3,4-thiadiazoles tested, two compounds (i.e. **3** and **4**) were found to be able to stabilize the DNA-topoII cleavable complex, i.e. to act as topoII poisons. However, the third of the active compounds (**2**) owes its anticancer effects to catalytic inhibition of topoII. From the pharmacological point of view, 2-(4-bromophenylamino)-5-(2,4-dichlorophenyl)-1,3,4-thiadiazole (**3**) seems to be the most promising. Not only did it influence the viability of both types of cancer cells in a more potent way than etoposide, but also had no toxic effects on normal cells (fibroblasts) within concentration ranges tested. The second topoII poison (**4**) recognized in the group of 1,3,4-thiadiazoles had a less selective effect on cancer cells. Compound **4** decreased the cells' viability and DNA synthesis in both cancer and normal cells. Having analyzed the results obtained, we managed to formulate some structure-activity relationships that might be helpful in designing novel topoII inhibitors/poisons based on 1,3,4-thiadiazole core. It is clear that the direct connection of the phenyl ring with 1,3,4-thiadiazole core at position 5 is essential for anticancer activity. The derivatives in which both aromatic systems were separated with –O–CH_2– linker (i.e. compounds **5** and **6**) were devoid of any activity and additionally inhibited DNA synthesis in normal cells. Nevertheless, the sole connection of both aforementioned aromatic rings (i.e. phenyl and 1,3,4-thiadiazole) is not a sufficient condition allowing anticancer effects to be evoked. The substitution pattern of a phenyl ring and the electronic character of substituents are also important. The replacement of chlorine atom (compound **1**) with a hydroxyl group (compound **2**) resulted in obtaining biologically active derivative. In the aforementioned case, it was evident that the effect of compound **2** on the viability of cancer cells resulted directly from the character of the hydroxyl group. This substituent is not only capable of forming strong hydrogen bonds with the active site of the enzyme, but also, as a potent electron-donating group, it should significantly change the electrostatic potential of a molecule. In spite of a low number of derivatives tested, it was possible to put forward a hypothesis that electrostatic potential of this fragment of the molecule affected the character of its interaction with human DNA topoII. Derivatives **3** and **4**, containing two electron-withdrawing substituents connected to the phenyl ring at position 5 of the 1,3,4-thiadiazole core, acted as topoII poisons whereas the presence of a potent electron-donating group (i.e. –OH) favored catalytic inhibition of topoII. Confirmation or refutation of this hypothesis requires further research involving a greater number of derivatives, which is currently being conducted. On the other hand, when considering the influence of a phenylamine group at position 2 of the 1,3,4-thiadiazole skeleton, it must be remembered that replacement of fluorine atom by bromine substituent improves anticancer activity. Thus, it might prove that substituents with higher volume and/or lower electronegativity are better tolerated in *para* position.

In order to give further insight into the correlations between anticancer activity of the studied compounds and their electronic properties, the frontier orbitals' distribution and electrostatic potential maps were calculated using DFT level of theory with B3LYP functional expressed on the basis set of 6-31G(d). The highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals, as well as the electrostatic potential maps were illustrated using the GaussView 5.0 program (Gaussian Inc., Wallingford, CT) (Figure S2). Contrary to what we expected, the replacement of chlorine atom in **1** with a hydroxyl group in compound **2** did not cause any significant changes in the electrostatic potential of the molecule. Similarly, the HOMO and LUMO distributions along the molecules were similar in the cases of compounds **1** and **2**. Thus, one can conclude that it is not electrostatics of the molecules but the particular properties of the hydroxyl group (e.g. the potential of forming hydrogen bonds), which is the source of such large differences in anticancer activity of the above-mentioned pair of compounds. In turn, a total lack of anticancer activity of compounds **5** and, **6**, containing –O–CH_2– linker, can be associated with a completely different geometry of the molecules and differences in HOMO/LUMO distributions. In the cases of the inactive derivatives (**5**, **6**) the HOMO orbitals are located at the 1,3,4-thiadiazole ring and the aromatic substituent in position **2** of this heterocyclic system, while for all active compounds the electron density of HOMO is virtually uniform around whole molecule. Again, the analysis of molecular orbitals distribution revealed that all active derivatives differ from the inactive ones (**5**, **6**) in low distribution of LUMO around the aromatic group in position 2 of the 1,3,4-thiadiazole ring. These observations clearly show that spatial distribution of the frontier molecular orbitals as well as geometry of the molecules are of greater importance for this class of compounds to exhibit anticancer activity.

Finally, there is one more fact that should be noted in relation to the anticancer activity of the studied compounds. Interestingly, the effect of compounds **3** and **4** on the viability of MCF-7 and MDA-MB-231 cells is different despite the fact that they cause nearly equivalent inhibition of DNA biosynthesis in both types of cancer cells. This might suggest that a total anticancer effect is also determined by other molecular mechanisms, apart from inhibition of intracellular DNA biosynthesis. This might also explain the absence of a linear relationship between the anticancer activity of compounds **1**–**6** and their docking scores obtained from preliminary docking studies.

Conclusions

To sum up, we have synthesized a series of 2,5-disubstituted 1,3,4-thiadiazole derivatives and evaluated them for anticancer and anti-topoisomerase activities. Interestingly, the active derivatives (**2**–**4**) present a better pharmacological profile than reference drug – etoposide. The latter inhibits the viability of fibroblasts by 50% at a concentration of 55 ± 2 μM whereas an analogous loss of MCF-7 and MDA-MB-231 cells viability was observed at the concentration 125 ± 2 μM and 98 ± 2 μM, respectively. On the other hand, we have observed no influence of compounds **2** and **3** on the viability of human normal skin fibroblasts in the concentration range tested with simultaneous reduction of breast cancer cells growth. Moreover, compounds **2**–**4** completely inhibited the activity of human DNA topoII in the concentrations twice as low as in the case of etoposide. Of the three active derivatives (**2**–**4**), 2-(4-bromophenylamino)-5-(2,4-dichlorophenyl)-1,3,4-thiadiazole (**3**) was found to exhibit the most promising biological effect. Its activity was even higher than that of the reference drug. It is worth pointing out that despite structural similarity of all three active 1,3,4-thiadiazole derivatives, the

molecular mechanisms of their anticancer activity were different, i.e. compound **2** was topoII catalytic inhibitor whereas two other derivatives acted as topoII poisons.

Declaration of interest

The authors declare no conflict of interest.

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Supplementary material available online
Supplementary Figures S1 and S2.