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Anticancer, neuroprotective activities and computational studies of 2-amino-1,3,4-thiadiazole based compound

Wojciech Rzeski,[a,c] Joanna Matysiak[b,*] and Martyna Kandefer-Szerszeń[a]

[a]*Department of Virology and Immunology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Lublin, Poland*
[b]*Department of Chemistry, Agricultural University, Lublin, Poland*
[c]*Department of Toxicology, Institute of Agricultural Medicine, Lublin, Poland*

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Abstract—Anticancer activity studies of 2-(4-fluorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (**FABT**), as one of the most promising derivatives from the *N*-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole set, have been continued. The tested compound inhibited proliferation of tumor cells derived from cancers of nervous system (medulloblastoma/rhabdosarcoma, neuroblastoma, and glioma) and peripheral cancers including colon adenocarcinoma and lung carcinoma. The anticancer effect of **FABT** was attributed to decreased cell division and inhibited cell migration. Furthermore, in anticancer concentrations it exerted a trophic effect in neuronal cell culture and had no influence on viability of normal cells including astrocytes, hepatocytes, and skin fibroblasts. Moreover, a prominent neuroprotective activity of **FABT** was observed in the neuronal cultures exposed to neurotoxic agents like serum deprivation and glutamate. To determine probability of tautomeric transition and indicate potential sites of interactions of **FABT** molecule with the receptor, quantum-chemical calculations with the ab initio Hartree–Fock model were made.
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1. Introduction

Despite significant progress achieved in anticancer therapy, the management of malignancies in humans still constitutes a major challenge for contemporary medicine.[1–4] Chemotherapy very often causes severe side effects, which are in part a consequence of destruction of normal cells. It was revealed that commonly used anticancer drugs cause significant toxicity in nervous system and are responsible for neurological side effects.[5–9] It is of crucial importance that anticancer drugs display antiproliferative activity in tumor cells without affecting normal tissues. Therefore, taking all the above-mentioned evidence into account, the development of novel chemotherapuetics and effective anticancer strategies is eagerly being pursued.

2-Amino-1,3,4-thiadiazole and its derivatives are well known as compounds of a wide range of anticancer activity,[10–15] including in vivo conditions.[16–18] For this type of derivatives a different mechanism of action is assigned, depending on the type of modification of 1,3,4-thiadiazole ring.[19–21] The action connected with the apoptotic mechanisms and angiogenesis, which is a crucial step in the tumorigenesis, seems to be very promising in anticancer therapy.[22,23] It was found that (*E*,*E*)-2,5-bis[4-(3-dimethylaminopropoxy)styryl]-1,3,4-thiadiazole induced the early-phase apoptosis in human non-small lung cancer A549 cells via the Bcl-X_L down-regulation, and that of the late-phase through up-regulation of Bax expression as well as inhibition of Akt/protein kinaze B (PKB). In addition, the compound showed equivalent anti-angiogenic activity in the nude mice angiogenesis model.[24]

In our previous publication we described synthesis and in vitro antiproliferative activity against some human cancer cell lines of compounds of 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole set.[25,26] The highest antiproliferative effect for *N*-halogenphenyl derivatives was found.[26] Therefore, we decided to extend studies on this type of compounds.

We herein report the further in vitro evaluation of 2-(4-fluorophenyloamino)-5-(2,4-dihydroxyphenyl)-1,3,

Keywords: 2-Amino-1,3,4-thiadiazole; Anticancer activity; Neuroprotective activity; Molecular descriptors.
* Corresponding author. Tel.: +48 81 4456816; fax: +48 81 5333752; e-mail: joanna.matysiak@ar.lublin.pl

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4-thiadiazole (**FABT**). The anticancer profile and neuroprotective activity of the compound are described. To determine probability of tautomeric transition and indicate potential sites of interactions of **FABT** molecule with the receptor, quantum-chemical calculations were carried out with the ab initio Hartree–Fock model.

2. Results and discussion

2.1. Biology

The antiproliferative effect of **FABT** (Fig. 1) was assessed in a range of human tumor cell lines. Tumor cells derived from cancers of nervous system (medulloblastoma/rhabdosarcoma, neuroblastoma, and glioma) and peripheral cancers including colon adenocarcinoma and lung carcinoma were tested. The cells were exposed to either culture medium (control) and **FABT** (5–100 μM) for 96 h. Proliferation of all tumor cell cultures was decreased in the cultures exposed to **FABT** (Fig. 2a) in a concentration-dependent fashion as measured by means of the MTT assay. Threshold concentrations of **FABT** required to elicit antiproliferative effect were as low as 5 μM (HT-29), 10 μM (A549, TE671), and 25 μM (SK-N-AS, C6). The effect of **FABT** on tumor cells was attributed to decreased cell division as determined by measurements of incorporation of BrdU during the DNA synthesis (Fig. 3). It is of crucial importance that anticancer drugs display antiproliferative activity in tumor cells without affecting normal cells. We have shown that normal cells were resistant to **FABT** up to 100 μM. Moreover, in the neuronal culture it showed a prominent neurotrophic activity (Fig. 2b). Chemotherapy very often induces severe side effects, which are in part a consequence of destruction of normal cells.[6] To evaluate the cytotoxic effect on normal cells, human skin fibroblasts (HSF) were subjected to increasing doses of **FABT** (5–250 μM). LDH assay revealed that **FABT** produced low cytotoxicity in normal HSF cell culture. The significant LDH release after 24 h exposure appeared at 100 μM (not shown). These data can suggest a beneficial side-effect profile of **FABT**.

The ability of tumor cells to migrate is one of the markers of tumor metastatic potential. To evaluate the effect of **FABT** on tumor cell motility, glioma (C6) and lung carcinoma (A549) cells were exposed to culture medium alone and 5 μM of **FABT** for 24 h. The wound assay revealed that in the cultures exposed to **FABT**, significantly fewer cells migrated to the wound area (Fig. 4a). The micrographs showing wound, cell migration in control culture, and inhibited glioma C6 cells migration following **FABT** exposure are presented in Figure 4b–d. The influence on tumor cell locomotion can suggest an antimetastatic potential of tested aminothiadiazole derivative.

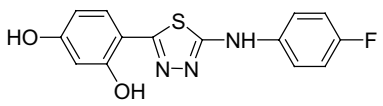

Figure 1. Chemical structure of 2-(4-fluorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (**FABT**).

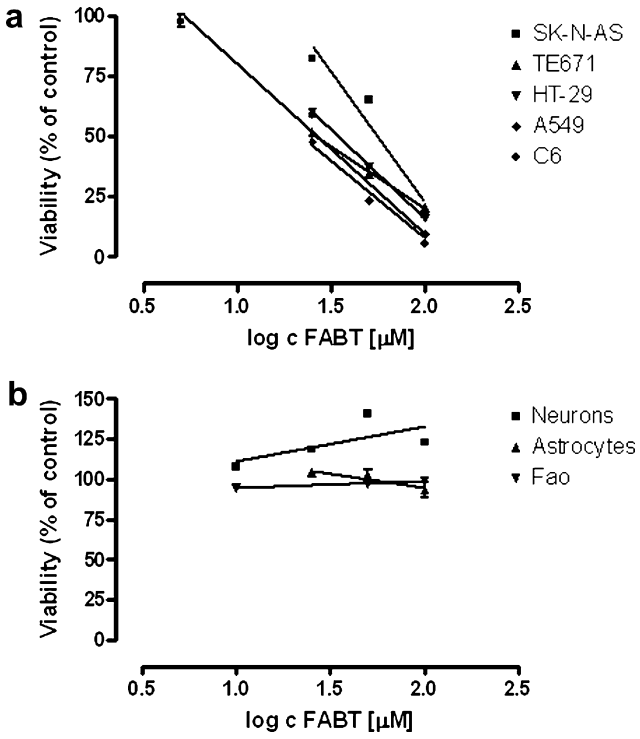

Figure 2. (a) Antiproliferative effects of **FABT** in cancer cells. **FABT** exerts a concentration (log*c*)-dependent antiproliferative effect in a range of tumor cell lines. Cells were exposed to either culture medium alone (control) and **FABT** (5–100 μM) for 96 h, and viability was measured by means of MTT assay. IC_{50}: SK-N-AS, 54.7 μM; TE671, 26.0 μM; HT-29, 33.1 μM; A549, 22.8 μM; C6, 27.3 μM. SK-N-AS, human neuroblastoma; C6, rat glioma; TE671, human rhabdomyosarcoma–medulloblastoma; A549, human lung carcinoma; HT-29, human colon adenocarcinoma; Fao, rat hepatocytes. (b) Effect of **FABT** on normal cells' viability. Rat cortical neurons, astrocytes, and hepatocytes (Fao) were exposed to either culture medium alone (control) and **FABT** (5–100 μM) for 48 h, and the viability was measured by means of MTT assay. The data represent mean normalized optical densities ±SEM of 5–8 trials and were analyzed by means of linear regression.

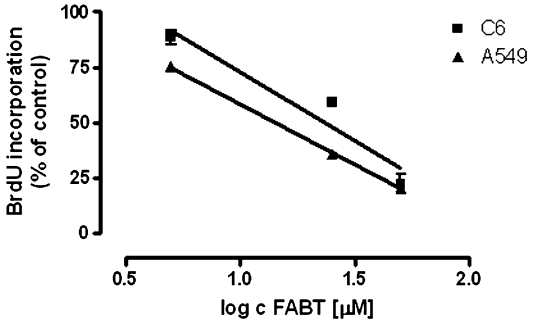

Figure 3. The antiproliferative effect of **FABT** was attributed to decreased cell division. Human lung carcinoma (A549) cells and rat glioma (C6) cells were grown in culture medium only (control) and in the presence of **FABT** (5–50 μM) for 48 h. BrdU incorporation was used as a marker of cell division. The data represent mean normalized optical densities ±SEM of 5–8 trials and were analyzed by means of linear regression. IC_{50}: C6, 23.5 μM; A549, 13.7 μM.

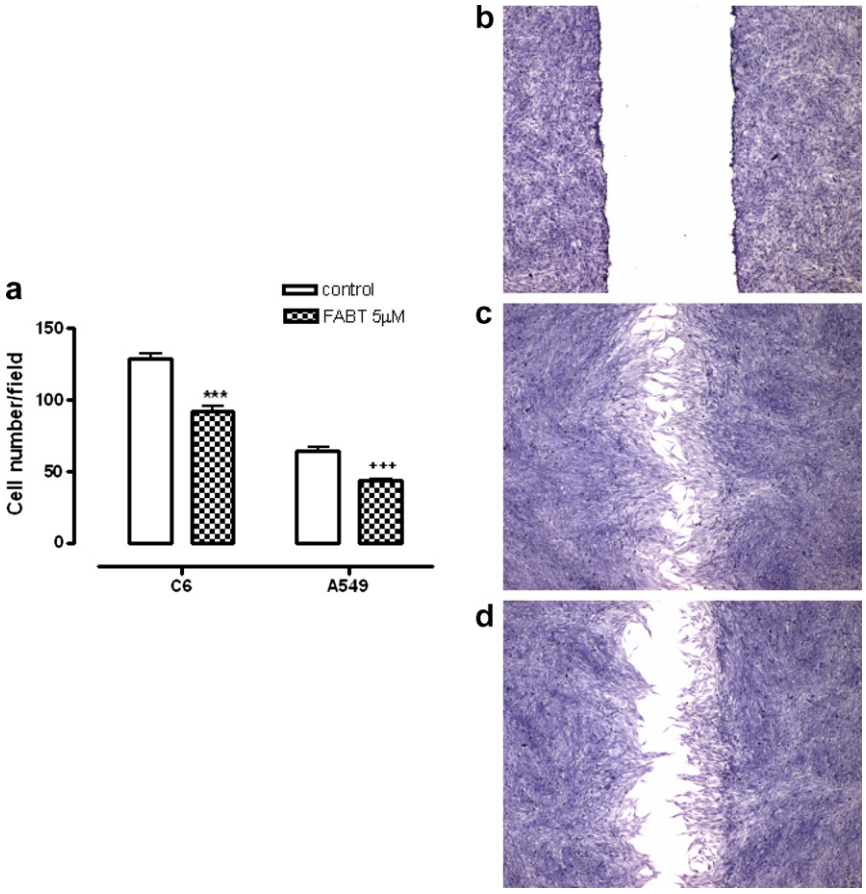

Figure 4. Effect of **FABT** on migration of tumor cells. Wounded monolayers of tumor cells (C6, A549) were incubated for 24 h alone or in the presence of **FABT** (5 μM). Tumor cells, which migrated to the wound area, were counted (a). Micrographs show wound assay of glioma C6 cells; wound (b), cell migration after 24 h in control culture (c) and following exposure to 5 μM **FABT** (d). Magnification 40×. The results are expressed as the mean number of cells migrated per field of the wound area ±SEM of 50 measurements; ***, $^{+++}$ at least $p < 0.001$ versus control, Student's t test.

Glutamate is the major neurotransmitter in the mammalian nervous system.[27] Abnormal glutamate signaling has been associated with neurological and psychiatric disorders including stroke, epilepsy, and neurodegenerative diseases, such as Alzheimer's, and Parkinson's disease.[28–30] In order to characterize the neuroprotective effects of tested compound, rat cortical neurons were exposed to the neurodegenerative agents like serum deprivation (SD) and glutamate (500 μM) alone and combined with **FABT** (10 and 25 μM). Both applied agents induced a prominent neurotoxicity which was further ameliorated by coexposure with **FABT** in a dose-dependent manner (Fig. 5). These preliminary data suggest that **FABT** exerts a prominent neuroprotective activity. Of note is the fact that concentrations of **FABT** required to elicit neuroprotection strongly influence tumor cell proliferation. In the nervous system, glutamate activates ionotropic and metabotropic receptors. Glutamate antagonists, which selectively block glutamate receptors, were demonstrated to have neuroprotective properties.[31] Recently, in a series of experiments we have described that these compounds elicit anticancer effects in vitro and in vivo.[32,33] The answer to the question whether **FABT** exerts glutamate antagonist properties will be a subject of further studies. Also, the in vivo studies and characterization of molecular mechanisms involved in **FABT** anticancer and neuroprotective activity remain to be done in order to evaluate its potential application as a new drug.

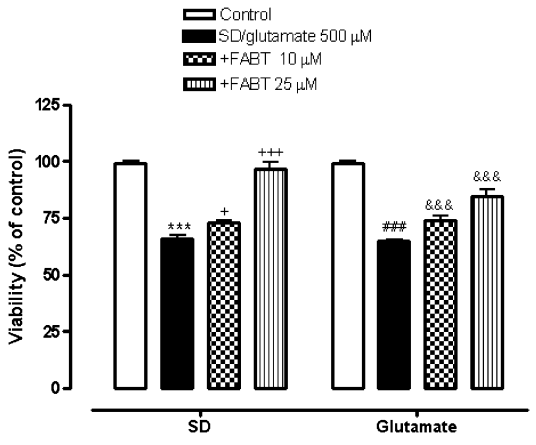

Figure 5. Neuroprotective effects of **FABT**. Neurodegeneration was induced by exposure of neuronal cell culture to serum deprivation (SD) and glutamate (500 μM). **FABT** (10, 25 μM) significantly ameliorates in vitro induced neurotoxicity. Columns represent mean normalized optical densities ±SEM of 4–8 trials. Statistical comparisons were performed between the results obtained with control, the neurotoxic agent alone, and those obtained in combination with **FABT** (+**FABT**). $^{***}p < 0.001$, $^{+}p < 0.05$, $^{+++}p < 0.001$ for exposure to SD; $^{\#\#\#}p < 0.001$, $^{\&\&\&}p < 0.001$ for exposure to glutamate; Student's t test.

2.2. Computational studies

Literature reports indicate existence of tautomeric forms of *N*-substituted 2-amino-1,3,4-thiadiazoles both in solutions and solid phase which can be of significant importance for biological activity.[34] The direction of equilibrium shift (Fig. 6) depends largely on substitution type of thiadiazole ring.

To determine probability of tautomeric transition and indicate potential sites of interactions of **FABT** molecule as one of the most active analogs from *N*-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole set, the quantum-chemical calculations were carried out with the ab initio Hartree–Fock model at the 6-31G** basis set. The determined values of HF energy (E_{HF}) of isolate molecule of both forms indicate thermodynamic preferences of the amine form A (Table 1). The additional indication is its smaller surface area, volume, and differences of heat formation (H_F) and energy solvatation (E_S) for both species. Existence of the imine form (B) for **FABT** was not confirmed in the determination conditions using spectroscopic methods. In spite of a relatively high energetic barrier of the tautomeric transition in the environmental conditions, after taking mainly solvatation process into account, the equilibrium coexisting imine form (B) cannot be excluded.

Molecular potential density distribution for amine structure (A) (Fig. 7) shows the most negative potential on nitrogen atoms of thiadiazole ring but the most positive on amine hydrogen atom. This may indicate capability of hydrogen bond formation with share of nitrogen atoms of the ring or through hydrogen atom of –NH- moiety. Negative potential is located on oxygen and fluorine atoms additionally. This is in agreement with LUMO and HOMO orbitals distribution of thiadiazole moiety (Fig. 8). LUMO involves sulfur and second carbon atom mainly, indicating the region reacting with potential biological nucleophiles. HOMO is located on nitrogen atoms (including amine atom) and represents the space of the greatest electron density. A large electron gap on C-2 compared to C-5 of 1,3,4-thiadiazole ring determined from the electrostatic potential distribution can also be essential in interactions with the receptor (Table 1). Experimentally large differences in electron density distribution for both mentioned carbon

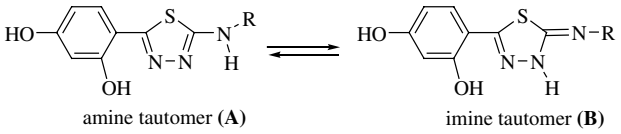

Figure 6. Tautomeric equilibrium for 2-amino-1,3,4-thiadiazoles.

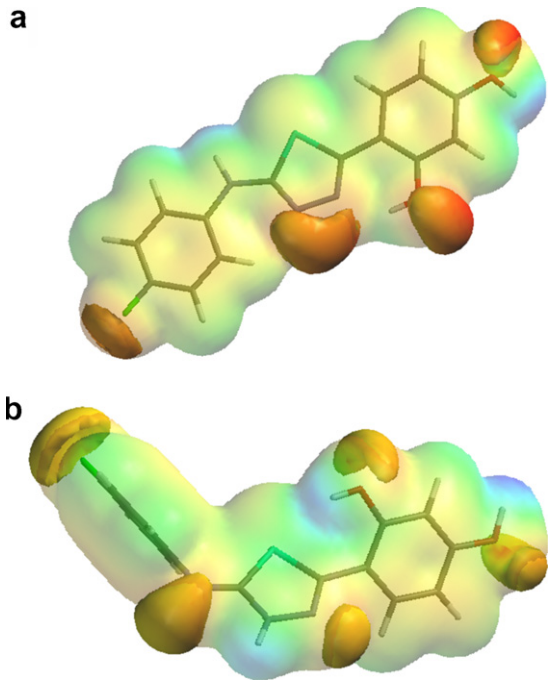

Figure 7. Molecular electrostatic potentials (MEPs) showing the most positive potential (deepest blue color), the most negative potential (deepest red color), and the intermediate potential (intermediate shades) regions. On MEPs the electrostatic potential profile at −20 kcal/mol was superimposed; (a) amine and (b) imine forms of **FABT**.

atoms are confirmed by position of resonance signals in the ^{13}C NMR.[25,26]

Assuming existence of tautomeric imine form (B) the analogous fragment of the molecule should be regarded as a potential site of interactions, whereby due to proton transfer, only one of nitrogen atoms would retain its previous properties of proton acceptor (N-4 of thiadiazole ring), but two would change their functions into reverse ones (Fig. 7b). Additionally, the electron gap on C-2 ring disappears. The charges of both carbon atoms of 1,3,4-thiadiazole ring become equal (Table 1).

Compound activity can be additionally intensified by the fragment of 2,4-dihydroxyphenyl, donor or acceptor of protons for hydroxyl groups. The *ortho*-position group of amine form (A) is involved in the intramolecular hydrogen bond which stabilized the planar conformation. This is confirmed by numerous literature reports on various analogs as well as by X-ray structural investigations by our team.[35] Besides direct interactions with the receptor, hydroxyl groups probably affect many other factors like transport, toxicity, essential for biological activity. Large potential accumulated on fluorine

Table 1. Structural, electronic, and thermodynamic properties of two species of **FABT**

Form	E_{HF} (kJ/mol)	Volume (Å^3)	Area (Å^2)	E_{LUMO} (eV)	E_{HOMO} (eV)	H_F (kcal/mol)	E_S (kJ/mol)	$q_{C\text{-}2}$ (e)	$q_{C\text{-}5}$ (e)
Amine (A)	−3533230.3	299.56	307.68	2.426	−7.862	−11.26	−17.39	0.70	0.31
Imine (B)	−3533192.5	302.43	314.03	2.870	−8.149	−9.92	−12.47	0.55	0.57

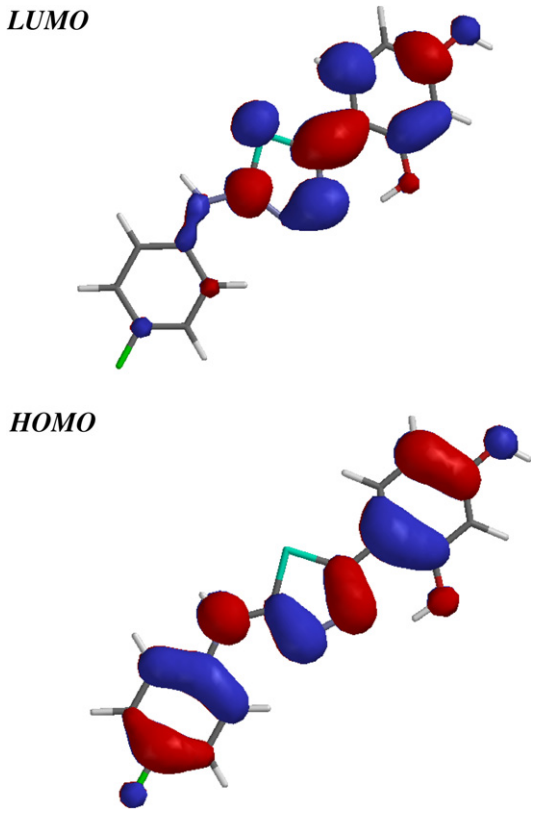

Figure 8. LUMO and HOMO isosurfaces for the amine form of **FABT**. Different surface colors represent opposite signs of the wavefunction. The structures are shown with a tube rendering; nitrogen atoms are in blue and sulfur atom is in green.

atom characteristic of both tautomeric forms can also play a significant role (Fig. 7).

The analysis indicates that independent of the form (though the amine form is probably predominant in the case of **FABT**), the moiety of three nitrogen atoms combined in the amino-five-membered system by means of sulfur atom is responsible for activity. Sulfur atom in a five-membered ring can affect relatively high aromatization of the system with relatively larger accessibility of free orbitals compared to analogous arrangements of atoms in the isostructural rings and intensifies electron density on nitrogen atoms due to greater tendency to pass into the electropositive state compared to other heteroatoms. It probably decides also about the required stericity, that is, bond length, angle size. Activity of this pharmacophore is, on one hand, intensified by the 2,4-dihydroxyphenyl substituent and on the other hand, by the *para*-fluorophenyl ring of exogenous nitrogen atom.

3. Conclusion

To sum up, we described herein the in vitro anticancer and neuroprotective activity of **FABT**. In a series of in vitro experiments we have shown that a new 2-amino-1,3,4-thiadiazole derivative elicits prominent anticancer effects in a range of tumor cell cultures. At the same time, the tested compound was not toxic to normal cells. Moreover, it possesses a prominent neuroprotective activity. Quantum-chemical calculations confirm the function of aminothiadiazole moiety as pharmacophore of this type of activity, intensified by the 2,4-dihydroxyphenyl substituent, independently of other properties of this benzenodiole moiety, and on the other hand, by the *para*-fluorophenyl substituent.

4. Materials and methods

4.1. Preparation of *FABT*

FABT (Fig. 1) was obtained from 4-(4-fluorophenyl)-3-thiosemicarbazide (Lancaster) and sulfinylbis(2,4-dihydroxythiobenzoyl) in the endocyclization process.[26] Mp: 279–280 °C; ^{1}H NMR (200 MHz, DMSO-d_6, δ): 10.85 (s, 1H, 2-COH), 10.27 (s, 1H, 4-COH), 9.92 (s, 1H, NH); IR (KBr, cm^{-1}): 3400, 3259, 3216 (OH, NH), 1629 (C=N), 1590 (C=C), 1231 (C–OH), 1183, 1134 (C–F), 1052 (N=C–S–C=N), 677 (C–S–C); EI-MS (*m*/*z*, %): 303 (M^+, 100) Anal. Calcd for $C_{14}H_{10}FN_3O_2S$ (303.32): C, 55.44; H, 3.22; N, 13.85. Found: C, 55.61; H, 3.23; N, 13.79.

4.2. Cell lines

Human rhabdomyosarcoma/medulloblastoma (TE671), human neuroblastoma (SK-N-AS), and rat hepatocytes (Fao) were obtained the from European Collection of Cell Cultures (Center for Applied Microbiology and Research, Salisbury, UK). Human Caucasian lung carcinoma (A549) and human colon adenocarcinoma (HT-29) were obtained from the Institute of Immunology and Experimental Therapy (Polish Academy of Sciences, Wrocław, Poland). Rat glioma (C6) was obtained from the Department of Neonatology, Charite-Virchow Clinics, Humboldt University, Berlin, Germany. Human skin fibroblasts (HSF) were a laboratory strain obtained by the outgrowth technique from skin explants of young persons.

The following culture media purchased from Sigma (Sigma Chemicals, St. Louis, MO, USA) were applied: DMEM (HT-29, C6, HSF), 1:1 mixture of DMEM and Nutrient mixture F-12 Ham (TE671, SK-N-AS), 2:1 mixture of DMEM and Nutrient mixture Ham's F-12 (A549), and Ham's F-12K (Fao). All media were supplemented with 10% FBS (Life Technologies, Karlsruhe, Germany), penicillin (100 U/mL) (Sigma), and streptomycin (100 μg/mL) (Sigma). The cultures were kept at 37 °C in humidified atmosphere of 95% air and 5% CO_2.

4.3. Neuronal cell culture

The neuronal cell culture was prepared from cortices of 18-day-old Wistar rat fetuses as previously described.[36] The tissue was pooled into ice cold glucose (33 mM) Hanks' Balanced Salt Solution (HBSS), cut into small pieces, and incubated for 30 min at 37 °C with 0.25% trypsin-EDTA solution. A single cell suspension was obtained by gentle pipetting of the cortex fragments in the

presence of 10% FBS and 0.01% DNase I (Sigma). The cells were then sieved through the 40 μM cell strainer (Falcon, Becton Dickinson Labware, Franklin Lakes, New Jersey, USA), centrifuged at 800 rpm for 10 min, and plated at 5×10^5 cells/mL density on poly-L-lysine (MW 70,000–150,000) coated 96-multiwell plates (Nunc, Roskilde, Denmark). The culture medium consisted of B-27 supplemented Neurobasal Medium (Life Technologies) which eliminates glial growth, 0.5 mM L-glutamine, and 1% of antibiotic-antimycotic solution (Life Technologies). For the initial plating the culture medium was supplemented with 25 μM glutamate. Incubation was carried out at 37 °C in humidified 95% air and 5% CO_2 atmosphere. The culture medium was changed every 3 days, until the culture reached 14 days. Neuronal identity was confirmed by positive staining with the mouse anti-neuron specific enolase γγ monoclonal antibody (Chemicon International, Inc., Single Oak Drive, Temecula, CA, USA). Antibody detection and antigen visualization were performed by using Streptavidin HRP Kit (STAR 2004) (Serotec Ltd, Oxford, UK).

4.4. Glial cell culture

The primary mixed glial cell culture was initially established according to the same protocol used for neurons. The cells were inoculated (15×10^6) into 75 cm^2 TC flasks and left for 48 h at 37 °C in 5% CO_2 incubator. Culture medium (1:1 mixture of DMEM and Ham F-12 nutrient mixture supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/mL streptomycin) was changed daily until the culture reached confluency (7–10 days). The flasks were shaken overnight in the orbital shaker at 210 rpm in order to remove less adherent cells (neurons, microglia, and oligodendroglia). Following this shaking procedure, the culture became enriched with flat cells displaying typical astrocyte morphology. Immunostaining with a primary antibody for glial fibrillary acidic protein (GFAP, polyclonal, DAKO, Denmark) revealed that astrocytes accounted for ~95% of the cells in the culture.

4.5. Viability assay

Tumor cells were plated on 96-well microplates (Nunc) at a density of 0.5×10^4 (C6), 1×10^4 (TE671, A549), and 3×10^4 (HT-29, SK-N-AS). Next day the culture medium was removed and the cells were exposed to serial dilutions of **FABT** in a fresh medium. Cell proliferation was assessed after 96 h by using the MTT method (Cell proliferation kit I, Boehringer Mannheim, Germany) in which the yellow tetrazolium salt (MTT) is metabolized by viable cells to purple formazan crystals. Tumor cells were incubated for 4 h with MTT solution (5 mg/mL). Formazan crystals were solubilized overnight in SDS buffer (10% SDS in 0.01 N HCl) and the product was quantified spectrophotometrically by measuring absorbance at 570 nm wavelength using E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Normal cells were plated on 96-well microplates (Nunc) at a density of 5×10^5 (neurons), 2×10^5 (astrocytes), and 2.5×10^5 (Fao). Next day the culture medium was removed and the cells were exposed to **FABT** (5–100 μM) for 48 h. Astrocytes and Fao cells were incubated in the medium containing 2% of FBS. Viability was assessed by means of MTT method.

In neuroprotection experiments, neurons were exposed to serum deprivation (SD—Neurobasal medium without B-27 supplement) and glutamate (500 μM) alone and combined with **FABT** (10 and 25 μM). Cell viability was assessed after 48 h by means of MTT method.

4.6. Proliferation assay

Cells were plated on 96-well microplates (Nunc) at a density of 1×10^4 (C6) and 2×10^4 (A549). Next day the culture medium was removed and the cells were exposed to serial dilutions of **FABT** in a fresh medium. Cell proliferation was quantified after 48 h by measurement of BrdU incorporation during DNA synthesis (Cell Proliferation ELISA BrdU, Roche Diagnostics GmbH, Penzberg, Germany). Tumor cells were incubated with 10 μM BrdU for 2 h. The cells were subsequently incubated with the FixDenat solution for 30 min and then exposed to monoclonal anti-BrdU antibodies conjugated to peroxidase. Color reaction was developed by adding the TMB substrate solution and terminated by addition of 1 M H_2SO_4. The absorbance was measured at 450 nm wavelength using E-max Microplate Reader.

4.7. Cytotoxicity assay

A cytotoxicity detection kit based on measurement of lactate dehydrogenase (LDH) activity was applied (Tox-7, Sigma). The assay is based on the reduction of NAD by the action of LDH released from damaged cells. The resulting NADH is utilized in stoichiometric conversion of a tetrazolium dye. The resulting colored compound is measured spectrophotometrically. Human skin fibroblasts (HSF) were plated on 96-well microplates at a density of 1×10^5. Next day the culture medium was removed and the cells were subjected to the tested compound (5–100 μM) diluted in a fresh culture medium with reduced amount of FBS (2%). Culture supernatants were collected after 24 h and incubated with substrate mixture for 30 min at room temperature in the dark. At the end, the reaction was terminated by the addition of 1 N HCl and the color product was quantified spectrophotometrically at 450 nm wavelength using E-max Microplate Reader.

4.8. Cell migration assessment

Tumor cell migration was assessed in the wound assay model. Tumor cells (C6, A549) were plated at 1×10^6 cells on 4-cm culture dishes (Nunc). Next day, the cell monolayer was scratched by the pipette tip (P300), the medium and dislodged cells were aspirated, and the plates rinsed twice with PBS. Next, the fresh culture medium was applied and the number of cells migrated into the wound area after 24 h was estimated in the control and the cultures treated with **FABT** (5 μM).

The plates were stained with the May-Grünwald–Giemsa method. The observation was performed in Olympus BX51 System Microscope (Olympus Optical CO., LTD, Tokyo, Japan) and the micrographs were prepared in analySIS® software (Soft Imaging System GmbH, Münster, Germany). Cells migrated to the wound area were counted on micrographs and results expressed as a mean cell number migrated to the selected 50 wound areas taken from four micrographs.

4.9. Computational methods

The compound was built with a standard bond length and angles using the PC SPARATN Pro Ver 1.08 molecular modelling program.[37] The energy was minimized by the molecular mechanic methods and then by the Hartree–Fock method at 6-31G**. Charge of atoms from the electrostatic potential distribution was determined.[38] Heat of formation was calculated using the PM3 method.

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