Neuroprotective activity of 2-amino-1,3,4-thiadiazole derivative 4BrABT – an in vitro study

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Abstract
4BrABT (2-(4-Bromophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole) is a compound known for its interesting in vitro anticancer profile. 4BrABT inhibited proliferation and motility of several cancer cell lines in concentrations which were not toxic to normal cells. A major problem associated with cancer chemotherapy, but also caused by environmental factors such as pesticides, is neurotoxicity. Therefore, the aim of the presented study was an in vitro evaluation of the neuroprotective activity of the compound. 4BrABT activity (1–100 μM) was tested in cultures of mouse neurons, rat astrocytes and rat oligodendrocytes. A possible protective action of the compound in different neurodegenerative models, as serum deprivation (SD), excitotoxicity (presence of 500 μM glutamate in culture medium), as well as cisplatin toxicity (astroglia – 50 μM and oligodendroglia – 100 μM) was investigated. Cell viability in the tested cultures was assessed with the use of LDH and MTT methods. Moreover, 4BrABT ability to prevent the cisplatin-induced apoptosis in astrocyte and oligodendrocyte cultures was analysed after Hoechst 33342 fluorostaining. The obtained results indicate that 4BrABT was not toxic to neurons, astrocytes and oligodendrocytes. Moreover, a decrease in the neuronal LDH level was observed, which may suggest the ability of 4BrABT to act as a trophic agent. Furthermore, the protective action of the studied compound was shown in neuronal cultures exposed to neurotoxic conditions (presence of glutamate and trophic stress) and in cisplatin-treated astrocytes and oligodendrocytes. The expression of anticancer and neuroprotective activity raises hopes for the potential use of 4BrABT as a safe anticancer drug, or neuroprotective agent in chemotherapy-associated neurotoxicity.

Key words
2-amino-1,3,4-thiadiazole, neuroprotection, neurodegeneration, excitotoxicity, trophic stress, neurotoxicity of cytostatics, neurotoxicity of pesticides

INTRODUCTION

The developments in medicine and improvement in living conditions have caused an increase of life expectancy. Thus, the human body, in particular the nervous system, is exposed to different disorders, such as Parkinson’s and Alzheimer’s diseases or senile dementia. A number of adverse conditions, including ischemia, hypoxia, hypoglycaemia, oxidative stress, brain injury, cytostatics used in chemotherapy, anaesthetic drugs and ethanol, may induce neurodegeneration [1, 2, 3, 4]. Moreover, it has been documented that many pesticides currently used in Europe, including organophosphates, carbamates, pyrethroids, ethylenebisdithiocarbamates, and chlorophenoxy herbicides, may cause neurotoxicity [5, 6, 7].

The adopted strategy of neuroprotection and regeneration processes depends not only on the type of induced changes, but also largely on the specific agent causing a current disorder. Frequently, it comprises the inhibition of disrupted signal transduction pathways, the usage of the glutamate receptor inhibitors, or neutralization of neurotoxic agent [4, 8, 9, 10, 11, 12].

Although numerous compounds have been proposed as neuroprotective agents, few have been proved to be effective against the chemotherapy-induced neurotoxicity. Thus, protection of the nervous system remains a challenge and a strategy for new drugs and treatment are needed to prevent neurodegenerative processes [4].

Therefore, the aim of the presented study was to evaluate the neuroprotective activity of 2-amino-1,3,4-thiadiazole derivative 4BrABT (2-(4-Bromophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole) (Fig. 1). Protective activity of the tested compound in different neurodegenerative models, including trophic stress, excitotoxicity, as well as cytostatic toxicity, was studied with the use of documented neurotoxic strategy – serum deprivation (SD) or neurotoxic strategy for new drugs and treatment are needed to prevent neurodegenerative processes [4].

Our previous studies demonstrated strong anticancer activity of the 2-amino-1,3,4-thiadiazole derivatives, including...
4BrABT in tumour cells derived from peripheral cancers, as well as cancers of nervous system origin [13, 14, 15, 16, 17]. 4BrABT in micromolar concentrations elicited a prominent antiproliferative effect in breast adenocarcinoma T47D, lung carcinoma A549, medulloblastoma/rhabdosarcoma TE671 and glioma C6 cells; the IC50 value was 7.7, 4.9, 12.5 and 13 μM, respectively. The anticancer effect was attributed to decreased DNA synthesis and was not connected with the induction of apoptosis [16]. It is significant that the tested 2-amino-1,3,4-thiadiazole derivatives in antiproliferative concentrations had no influence on normal cells viability, including skin fibroblasts, hepatocytes, neurons and astrocytes [13, 16, 17]. Moreover, prominent neuroprotective activity of the 2-(4-Fluorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (FABT) was observed in the neuronal cultures exposed to neurotoxic conditions, including serum deprivation and presence of glutamate [13]. Importantly, aminothiadiazoles were observed to readily penetrate the blood–brain barrier [18, unpublished data], which creates the possibility of their clinical application.

MATERIALS AND METHOD

Cell cultures. Experiments were carried out on mouse neurons, primary rat astrocytes and rat oligodendrocytes (OLN-93). Neuronal cell cultures were prepared from retinoic acid-induced neural differentiation of P19 mouse embryonal carcinoma cells (ATCC, American Type Culture Collection, Manassas, VA, USA). Rat astroglia was isolated from the brains of the suckling rat pups. Oligodendrocytes cell line (OLN-93) was obtained from the Department of Neonatology, Charité-Virchow Clinics, Humboldt University, Berlin, Germany.

Astrocytes and oligodendrocytes were cultured in 1:1 mixture of DMEM and Nutrient mixture F-12 Ham (Sigma Chemicals, St. Louis, MO, USA), P19 teratoma in AlphaMEM (Gibco, Life Technologies, Milan, Italy) and neurons in Neurobasal medium (Gibco). Media were supplemented with 10% FBS (Sigma), penicillin (100 U/mL) (Sigma) and streptomycin (100 μg/mL) (Sigma). Neurons were cultured in medium with the addition of 2% B12 supplement (Gibco). The cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Neuronal cell culture. P19 cells were cultured in AlphaMEM with 10% FBS. For induction of the neuronal differentiation, cells were resuspended in AlphaMEM medium supplemented with 5% FBS and 0.5 μM retinoic acid at a density 1 × 10⁵ cells/mL. Cells were incubated for 96 h on Petri dishes (the medium was changed for fresh one after 48 h). The obtained neurospheres were aspirated and centrifuged at 30 x g for 5 min, rinsed with serum free medium and centrifuged again. To obtain a single cell suspension, neurospheres were treated for 10 min in 37°C with 0.01% trypsin-EDTA and 0.01% DNAase I solution. The cells were then sieved through a 40 μm cell strainer (Falcon, Becton Dickinson Labware, Franklin Lakes, New Jersey, USA). Finally, the cell suspension was centrifuged at 100 x g for 10 min and resuspended in culture medium containing Neurobasal medium (Gibco), 2% B-27 supplement (Gibco), 2 mM L-glutamine (Sigma) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The cells were plated at a density of 4 × 10⁴ cells/mL on 96-well microplates (Nunc) coated with poly-L-lysine (Sigma) and kept at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The culture medium was changed every 3 days. The experiments were carried out 10 days after culture establishment.

Astroglia culture. The astrocyte primary cultures were established from the cortices of 3-day-old Wistar rats. The brain tissue was pooled into ice cold glucose (33mM) Hanks’ Balanced Salt Solution (HBSS, Sigma), cut into small pieces and incubated for 30 min at 37°C with 0.25% trypsin-EDTA solution. Single cell suspension was obtained, as described above for neuronal culture. The cells were inoculated into 75 cm² cell culture flasks and kept at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The culture medium consisted of a 1:1 mixture of DMEM and Nutrient mixture F-12 Ham supplemented with 10% FBS and antibiotics (penicillin and streptomycin). Medium was changed every 2 days until the culture reached confluency. Next, flasks were shaken overnight in orbital shaker at 210 rpm in order to remove less adherent cells (neurons, microglia and oligodendroglia). Following this shaking procedure, 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.

Viability assay. Cells were plated on 96-well microplates (Nunc) at a density 1 × 10⁴ (astrocytes, OLN-93) and 4 × 10⁵ (neurons) cells/mL. The following day, the culture medium was removed and the cells exposed to serial dilutions of 4BrABT (1, 2.5, 5, 10, 25, 50, and 100 μM), 500 μM glutamate (Sigma) and cisplatin (50 and 100 μM) alone or in combination in a fresh medium with 2% of FBS (astrocytes, OLN-93). Cell viability was assessed after 24 h (astrocytes and OLN-93) and 48 h (neurons) by means of the LDH and MTT method.

LDH method. The In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma) was applied. The assay is based on the reduction of NAD by lactic dehydrogenase (LDH) released from damaged cells. The resulting NADH is utilized in stoechiometric conversion of a terazolium dye. The resulting coloured compound is measured spectrophotometrically. The test was carried out according to the kit procedure. The colour product was quantified spectrophotometrically at 490 nm wavelength using an Elx800 microplate reader (BIO-TEK, Highland Park, Winooski, Vermont, USA).

MTT method. In the MTT method the yellow tetrazolium salt (MTT) is metabolized by viable cells to purple formazan crystals. The cells were incubated for 3–4 h with MTT solution (5 mg/mL). Formazan crystals were solubilized overnight in SDS buffer (10% SDS in 0.01N HCl) and the product quantified spectrophotometrically by measuring absorbance at the 570 nm wavelength using an Elx800 microplate reader. The In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma) was applied. The assay is based on the reduction of NAD by lactic dehydrogenase (LDH) released from damaged cells. The resulting NADH is utilized in stoechiometric conversion of a terazolium dye. The resulting coloured compound is measured spectrophotometrically. The test was carried out according to the kit procedure. The colour product was quantified spectrophotometrically at 490 nm wavelength using an Elx800 microplate reader.

Fluorescent cell death analysis. Astrocytes and oligodendrocytes were plated on Lab-Tek Chamber Slide (Nunc). The next day, cells were exposed to 4BrABT and cisplatin alone and jointly. Due to differences in the sensitivity of astroglia and oligodendroglia to cisplatin and...
the tested compound optimal concentrations were selected. The concentrations of tested compounds were used as follows: 25 μM 4BrABT and 50 μM cisplatin for astroglia or 10 μM 4BrABT and 100 μM cisplatin for oligodendroglia. After 24 h treatment, fluorescent dye Hoechst 33342 (final concentration 2 μg/ml) was added to cell cultures and incubated 5 min at 37°C. Then pictures were captured in fluorescent microscope (Olympus Optical Co., Ltd, Tokyo, Japan) with 420 nm UV filter and prepared by analysis software (Imaging Software Olympus cell*Family).

**Data analysis.** One-way ANOVA test, post-hoc Tukey was applied for statistic analysis with use of GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

**RESULTS AND DISCUSSION**

Synthesis and structural formula of 4BrABT have been described previously [16]. The effect of 4BrABT in central nervous system cells, including mouse neurons, rat astrocytes and rat oligodendrocytes, was tested. Cells were treated with the compound in concentrations ranging from 1–100 μM for 24 h (astrocytes and oligodendrocytes) or 48 h (neurons), then LDH and MTT assays were performed. Results demonstrated lack of the toxicity of 4BrABT in cell cultures derived from nervous system (Fig. 2). Moreover, a statistically significant decrease of the LDH level in the range 5–100 μM in neuronal, 1–25 μM in astrocyte and 10–100 μM in oligodendrocyte cultures was shown. MTT test results confirmed these findings (data not shown). Obtained data may suggest the neuroprotective effect of tested derivative.

In order to characterize the neuroprotective activity of 4BrABT, mouse neuronal cultures were exposed to glutamate (500 μM) and SD alone and combined with the tested compound. Both applied agents induced a prominent neurotoxicity which was further suppressed by co-exposure with 4BrABT (Fig. 3). Glutamate (500 μM) induced strong toxicity in tested neuron culture. Simultaneously, in cultures treated with 4BrABT, a prominent decrease of the LDH level was noted. A statistically significant protective effect was observed at concentrations 10 and 25 μM of 4BrABT (Fig. 3A). The neuroprotective activity of the tested compound was also established with respect to cells subjected to SD. The absence of the trophic factors in culture medium caused no damage to the cell membrane; therefore, the MTT test was applied to determine cell viability. The significant protective effect, observed as an increase in metabolic activity, was shown already at 5 and 10 μM of 4BrABT.

At the concentration of 10 μM, additional neurotrophic activity of the tested compound was reported as an increase of the cell viability above the level of that in the control culture (Fig. 3B). Discussed results presenting the protective effect of 4BrABT in neurons under altered conditions which occur in neurodegeneration, such excitotoxicity and lack of trophic factors remains in agreement with data obtained by Rzeski et al., revealed for fluoro-derivative of 2-amino-1,3,4-thiadiazoles FABT [13].

The neuroprotective activity is important in the context of brain tumours due to glutamate release from glioma cells which may actively affect surrounding neuronal cells [19]. The demonstrated ability to protect neurons against glutamate might suggest potential action of 4BrABT through glutamate receptors. Taking into consideration the expression of glutamate receptors on the surface of cancer cells, this mechanism of action could be beneficial for antitumour and neuroprotective substances [20]. However, more research is needed to confirm the stated hypothesis.

Moreover, the effect of 4BrABT in astroglia and oligodendroglia exposed to cytostatics was tested. Influence of the 4BrABT and cisplatin alone or in combination on the cell viability, as well as the level of cell death (apoptosis) in cultures was analyzed.

In the astroglia culture, cisplatin induced a prominent decrease of the cell viability, which was abolished as a result of 4BrABT action (Fig. 4A). Simultaneously, the effect was confirmed by Hoechst 33342 staining (Fig. 4B-E). Apoptosis in astroglia culture treated with cisplatin (50 μM) was observed as bright, shining clusters of lumps (cells with condensed chromatin and apoptotic bodies) (Fig. 4D), while
in cultures treated with 4BrABT the effect was diminished (Fig. 4E). A greenish glow seen in cells treated with the tested compound is suggested to be a non-specific effect of light shining on the substance under UV light (Fig. 4C). In astroglia culture treated both with cisplatin and 4BrABT, a significantly lower level of apoptosis was observed, in comparison to cisplatin (Fig. 4E).

Similarly, in oligodendrocyte OLN-93 cells which were found to be much more resistant to the toxic effects of cisplatin than astrocytes, which was confirmed by Dietrich et al. [21]. Viability of oligodendrocytes decreased after cisplatin treatment (100 μM), but co-incubation with 4BrABT significantly raised the level of actively metabolizing cells (Fig. 5 A). Moreover, the proapoptotic effect of cisplatin observed in OLN-93 cells treated with this cytostatic was abolished by 4BrABT (Fig. 5B-E). Glia cells provide support and protection for neurons, thus, their damage by cytostatics may indirectly influence on neuronal viability and functions.

Interestingly, our previous studies confirmed that both 4BrABT [16] and FABT [13] exerted antiproliferative activity against several cancer cell lines, including glioma cells. These derivatives inhibited cancer cell proliferation, DNA synthesis...
and cell migration. However, the differences between 4BrABT and FABT in effectiveness of antiproliferative activity was observed. The IC50 value for FABT (A549, 22.8 µM; C6, 27.3 µM; TE671, 26 µM) was significantly higher compared with 4BrABT.

For the first time, the presented study reports the neurotrophic and neuroprotective properties of 4BrABT. Importantly, in contrast to several substances exerting antiproliferative activities, 4BrABT did not induce apoptosis in central nervous system cells. On the contrary, it protected astrocytes and oligodendrocytes against cisplatin-induced apoptosis. The presented data discusses the thesis that anticancer agents are potent neurotoxin in vitro and in vivo [22]. 4BrABT did not induce toxicity in central nervous system cells; moreover, it exerted a protective effect on these cells. This hallmark may underlie future application of 4BrABT in cancer chemotherapy.

CONCLUSIONS

Anticancer agent 4BrABT revealed prominent neurotrophic and neuroprotective activity in central nervous system cells, including neurons, astrocytes and oligodendrocytes. Taking into consideration the neuroprotective and previously documented anticancer activity, 4BrABT may be potentially considered as a new therapeutic in cancer chemotherapy. Moreover, its application with commonly used cytostatics may decrease chemotherapy-associated neurotoxicity.

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