Abstract. Pancreatic cancer is one of the deadliest malignancies characterized by strong resistance to almost all chemotherapeutic agents and radiotherapy. In this study, we aimed to investigate the anticancer effect, enzymatic antioxidant activity [superoxide dismutase (SOD), glutathione peroxidase (GPx)] and total antioxidant capacity (TAC) of synthesized benzothiazole compounds against adenocarcinoma cancer cells (PANC-1). 2-((1S,2S)-2-((E)-4-nitrostyryl)cyclopent-3-en-1-yl)benzo[d]thiazole and 2-((1S,2S)-2-((E)-4-fluorostyryl)cyclopent-3-en-1-yl)benzo[d]thiazole containing 2-substituted benzothiazole group were synthesized in two steps. PANC-1 cells were treated with different concentrations of benzothiazole compounds (5, 25, 50, 75 and 100 μM) for 48 h and their cytotoxicity effects were determined by the MTT assay. To determine whether these compounds induced apoptosis, PANC-1 cells were treated with increasing concentrations of the synthetic products. Our study showed that the synthesized compounds have antiproliferative effects against PANC-1 cells and reduced cell viability. These compounds induced apoptosis of pancreatic cancer cells and at the same time reduced the activity of SOD and GPx and reduced TAC. On the basis of these findings, these synthesized benzothiazole compounds may be considered as a potential therapeutic drug against human PANC-1 cancer cells.

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developed with minimum side-effects to prevent and treat cancer (11). In this context, we describe the synthesis, spectroscopic data, antioxidant and antiproliferative activity against PANC-1 pancreatic cancer cells of 2-substituted benzothiazoles 2-((1S,2S)-2-((E)-4-nitrostyryl)cyclopent-3-en-1-yl)benzo[d]thiazole and 2-((1S,2S)-2-((E)-4-fluorostyryl)cyclopent-3-en-1-yl)benzo[d]thiazole. We examine dose-dependent antioxidant properties of these synthesized benzothiazoles against PANC-1 cell line in vitro, taking into consideration the important role of ROS in cancer formation.

Materials and Methods

Materials. All the reagents and chemicals were supplied by Sigma-Aldrich (Darmstadt, Germany) and Merck (Darmstadt, Germany) and were used without purification. Melting points of synthesized compounds were measured with Electrothermal 9100 apparatus (Bibby Sci. Ltd, Stone, Staffordshire, UK). Reactions were controlled by using pre-coated silica gel aluminum backed thin layer chromatography (TLC) Kiselgel plates (Merck) 60 F254 Merck in hexane:ethyl acetate (95:5). Column chromatography was performed for purification with Silica Gel 60. 230–400 meshes (hexane:ethyl acetate). Infrared spectroscopy (IR) spectra were recorded by a PerkinElmer FT-IR Spectrometer 400 (Waltham, MA, USA) (resolution: 0.5-4 cm⁻¹). ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance III instrument (Billerica, MA, USA) using deuterated chloroform (CDCl₃) as solvent. Elemental analyses (C, H, N, S) were performed using a LECO CHNS 932 elemental analyzer (St. Joseph, MI, USA). UV-Visible absorption measurements were performed using a Shimadzu TCC-240A spectrophotometer (Kyoto, Japan) with 1 cm length quartz cells. Enzyme-linked immunosorbent assay (ELISA) measurements were carried out in a Thermo Scientific Multiskan FC (Vantaa, Finland) microplate reader using 96-well microtiter-plates.

Synthesis of chalcone derivatives. Chalcone derivatives were synthesized based on Claisen–Schmidt condensation as previously reported (12). NaOH was added to a powerfully stirred solution of cis-bicyclo[3.2.0]hept-2-en-6-one (1 in Figure 1) and aldehyde derivatives (2 in Figure 1). The resulting solution was stirred for 4 hours at room temperature. Chloroform was added to the mixture and the organic phase was washed, dried with sodium sulfate and filtered. Solid compound was recrystallized in ethyl acetate/hexane (1:9). The obtained compound 3a and 3b were used in the next step (Figure 1).

(E)-7-(4-Nitrobenzylidene)bicyclo[3.2.0]hept-2-en-6-one (3a): Yield: 87%; mp: 166-170°C; ¹H-NMR (400 MHz, CDCl₃): δ=8.28 (d, J=8.8 Hz, 2H), 7.74 (d, J=8.8 Hz, 2H), 6.89 (s, 1H), 6.00-5.94 (m, 2H), 4.48-4.46 (m, 1H), 3.92-3.88 (m,1H); 2.78 (bd, J=17.6 Hz, 1H), 2.58 (dd, J=17.6, 10.4 Hz, 1H). ¹³C-NMR (100 MHz, CDCl₃): δ=203.3, 153.1, 147.9, 140.6, 134.4, 130.2 (2 C), 127.7, 124.2 (2 C), 121.2, 61.3, 49.9, 35.1.

(E)-7-(4-Fluorobenzylidene)bicyclo[3.2.0]hept-2-en-6-one (3b): Yield: 82%; mp: 80-84°C; ¹H-NMR (400 MHz, CDCl₃): δ=7.59 (dd, J=8.8, 5.6 Hz, 2H), 7.12 (t, J=8.8 Hz, 2H), 6.84 (d, J=2.0 Hz, 1H), 6.03-6.00 (m, 1H), 5.90-5.88 (m, 1H), 4.38-4.37 (m, 1H), 3.95-3.92 (m,1H); 2.83-2.77 (dm, J=15.2 Hz, 1H), 2.64-2.56 (dd, J=15.2, 8.8, 2.9 Hz, 1H). ¹³C-NMR (100 MHz, CDCl₃): δ=203.7, 164.8, 162.3, 148.9, 133.6, 131.8, 131.7, 130.5, 130.4, 128.5, 123.0, 116.4, 116.1, 60.7, 49.4, 34.7.

Synthesis of benzothiazole derivatives. Benzothiazole compounds were synthesized from chalcone compounds 3a and 3b (1.0 equivalent) then reacted with 2-amino-thiophenol (1.0 equivalent) in ethanol (50 ml) in the presence of p-toluene sulfonic acid. The mixture was then refluxed for 10 hours, monitoring by TLC and then cooled. Chloroform was added to the reaction mixture and the organic phase was washed, dried with sodium sulfate and filtered. The final products (4a and 4b) were purified by chromatography with hexane and an increasing amount of ethyl acetate (0-15%) yielding a waxy solid.

2-((1S,2S)-2-((E)-4-nitrostyryl)cyclopent-3-en-1-yl)benzo[d]thiazole (4a): Yield: 82%; ¹H-NMR (400 MHz, CDCl₃): δ=8.18 (d, J=8.1 Hz, 1H), 8.01 (d, J=8.1 Hz, 1H), 7.88 (d, J=8.0 Hz, 1H), 7.61-7.44 (m, 3H), 7.39 (t, J=7.6 Hz, 1H), 6.64-6.47 (m, 2H), 6.06-5.95 (m, 1H), 5.85-5.76 (m, 1H), 4.01 (ddt, J=6.9, 4.5, 2.2 Hz, 1H), 3.80 (dt, J=8.8, 7.4 Hz, 1H), 3.12 (ddt, J=13.3, 6.7, 2.2 Hz, 1H), 3.02-2.89 (m, 1H). ¹³C-NMR (100 MHz, CDCl₃): δ=174.3, 153.0, 146.7, 143.6, 136.6, 134.9, 131.6, 130.9, 128.8, 123.0, 116.4, 116.1, 60.7, 49.4, 34.7.
Cell culture. PANC-1 cells were obtained from the American Tissue Culture Collection (Manassas, VA, USA). Cells were cultured as described previously (13). In brief, cells were cultured in high glucose Dulbecco’s modified Eagles medium (DMEM) (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies) at 37°C in a humidified atmosphere of 5% CO₂. Media were replaced every 2-3 days. For 3 days prior to the experiment, PANC-1 cells were cultured in the absence of synthesized benzothiazole compounds (0 μM). For 3 days, cell viability was assessed by MTT assay (Sigma). Cells were seeded into 96-well transparent flat bottom plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 1×10⁵ cells/well for 24h. After 24h of incubation at 37°C, the medium was removed and the cells were treated with synthesized benzothiazole compounds (0, 5, 25, 50, 75 and 100 μM). The plate was incubated at 37°C for 48 h after which the medium was removed. Then, 200 μl of MTT reagent (1 mg/ml in serum-free medium) was added to each well. After 4hours, the medium was removed and 200 μl dimethyl sulfoxide (DMSO) was added to each well. The metabolized MTT product dissolved in DMSO was quantified by reading the absorbance at a wavelength of 570 nm on a micro plate reader (Thermo Scientific Multiskan). We used gencitabine, a chemotherapeutic agent used against pancreatic carcinoma (14), as positive control and untreated cells (0 μM) as negative control for cell toxicity. The 50% inhibitory concentration (IC₅₀) values were calculated and the IC₅₀ curves were plotted using GraphPad Prism 3.0 (La Jolla, CA, USA) based on a sigmoidal dose–response equation.

Apoptosis assay. PANC-1 human pancreatic cells were seeded in flat-bottom plates and after 24 h of incubation at 37°C, the medium was removed and the cells were treated with synthesized benzothiazole compounds (0, 5, 25, 50, 75 and 100 μM). The plate was incubated at 37°C for 48 h. Cells were lysed, and measurement of DNA degradation was estimated using the cell death detection ELISAPLUS kit (Roche Applied Science, Germany) following the manufacturer’s protocol. Absorbance was determined at 405 nm. The enrichment factor was calculated as the ratio of the absorbance of the sample cells to the absorbance of control cells as described elsewhere (15). An enrichment factor of 1 or more represents background or spontaneous apoptosis.

Determination of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. PANC-1 human pancreatic cancer cells were treated with different concentrations (0, 5, 25, 50, 75 and 100 μM) of synthesized benzothiazoles and incubated for 48 h. After the incubation period, the medium was removed and cell pellets were then lysed in phosphate buffer (PBS, pH 7.0), followed by sonication for 2 min on ice. To obtain supernatant, the mixture was centrifuged at 14,000 × g for 10 min and assayed for enzyme activities and protein concentration. Protein levels were calculated by the Lowry method (16). SOD activity was determined with Fridovich method (17). The GPx assay was based on the oxidation of NADPH to NADP⁺, which leads to a decrease in absorbance at 340 nm. The rate of this decline is directly proportional to the GPx activity in the sample (18).

Results

Chemistry. The target compounds were obtained in two steps, as described above (Figure 1). Compounds 4a and 4b were synthesized from reaction between 2-amino-thiophenol with chalcones 3a and 3b in ethanol at reflux (10 h) in good yield (Table I). All spectral data of compounds 4a and 4b ((¹H NMR, ¹³C NMR, IR, elemental analysis) corresponded with the proposed structures.

Cytotoxicity against PANC-1 cancer cell line. The cytotoxic effect of benzothiazole derivatives (4a and 4b) on PANC-1 human pancreatic cancer cells was determined by MTT assay after treatment with 5 to 100 μM of the compounds for 48 h. Treatment with increasing concentrations of synthesized benzothiazole compounds (0, 5, 25, 50, 75 and 100 μM) led to a concentration-dependent inhibition of cell growth (Figure 2). While gencitabine had a half-maximal inhibitory concentration (IC₅₀) of 52±0.72 μM for PANC-1 cells, the synthesized benzothiazole compounds had IC₅₀ values of 27±0.24 μM (4a) and 35±0.51 μM (4b). The inhibitory effect of the synthesized benzothiazole compounds on pancreatic cancer cells was greater than that of gencitabine.
Cell apoptosis assay. Although cytotoxicity and cell viability is often determined by MTT assay, this assay does not differentiate between cell death from apoptosis and necrosis. We performed an apoptosis assay to determine if the cytolysis of PANC-1 cells after treatment with synthesized benzothiazole compounds is apoptotic. This assay provides a quantitative determination of cytoplasmic histone-associated DNA fragments stemming from DNA degradation that result specifically in apoptotic cells. As shown in Figure 3, the rate of apoptotic cells increased in a concentration-dependent manner when treated with synthesized benzothiazole compounds. Treatment of cells with 100 μM or 75 μM compound 4a for 48 h resulted in increased number of apoptotic cells ranging from 1.35- to 1.51-fold over that the control (0 μM). Also, in comparison with the controls (0 μM), the rate of apoptotic cells in PANC-1 culture was 1.35 fold higher after incubation with compound 4b at 100 μM. These results demonstrate that treatment with synthesized benzothiazole compounds induced apoptosis of PANC-1 cells in vitro.

Effect on SOD and GPx activities, and TAC. In general, all concentrations (5, 25, 50, 75, 100 μM) of 4a and 4b effectively reduced SOD and GPx activity in treated pancreatic adenocarcinoma cells compared to untreated control cells. For SOD activity, the decrease was significant at 50, 75 and 100 μM 4a when compared to the control (Figure 4A). Among the five doses, 75 and 100 μM caused greater decreases in GPx level (Figure 4B). Compound 4b effectively reduced the activities of SOD and GPx in PANC-1 pancreatic cancer cells compared with the control group. The decrease in SOD activity was greater at 75 and 100 μM doses when compared to the control group (Figure 5A). With respect to GPx levels, benzothiazole compounds significantly strongly reduced GPx at 75 and 100 μM concentrations (Figure 5B) compared to the control group.

TAC was also determined in culture supernatants. Figure 4C and 5C show that there were statistically significant differences in TAC values between untreated PANC-1 cells and those that were treated with synthesized benzothiazole compound. Among the five doses, 25, 50 and 100 μM doses of compound 4a caused greater decrease in TAC (Figure 4C). On the other hand, 4b led to a greater decrease at 100 μM concentration (Figure 5C) compared to the other treatment groups.

Discussion

Benzothiazole is a privileged heterocyclic scaffold possessing diverse pharmaceutical applications. The potent antitumor activities displayed by various 2-arylbenzothiazole derivatives have reinforced their importance in the development of newer and effective antitumor agents. Moreover, broad-spectrum antimicrobial activity demonstrated by benzothiazole derivatives make them agents of choice for the development of antimicrobial lead drugs. Furthermore, promising anticancer, anti-inflammatory, neuroprotective, antidiabetic, analgesic and many other activities demonstrated by molecules containing a benzothiazole nucleus make it a much sought after scaffold for the development of corresponding therapies. In recent years, a large number of patents have been filed discussing various pharmacological properties of benzothiazoles, signifying the potential of this scaffold for the development of new chemical entities in the treatment of diseases/disorders (20).

Tumorigenesis is ascribed to uncontrolled proliferation as well as induced ROS and reduced p53-dependent apoptosis (21). Medicinal and pharmaceutical chemistry have been a

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<th>Product</th>
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substantial source from which great number of apoptosis-inducing agents have been obtained (11). The induction of apoptosis in tumor cells is the most extensive mechanism of anticancer drug action in many cancer therapies (22). Recent studies revealed that benzothiazole derivatives possess cytotoxic and antitumor effects on several tumor cell lines in vitro (20, 23) and in vivo (24). Our previous study showed that benzothiazole compounds dose-dependently inhibit the proliferation of C6 (rat brain tumor) and HeLa (human uterine cancer) cell lines (25). These results are supported by our present finding that compounds 2-((1S,2S)-2-((E)-4-nitrostyryl)cyclopent-3-en-1-yl)benzo[d]thiazole (4a) and 2-((1S,2S)-2-((E)-4-florostyryl)cyclopent-3-en-1-yl)benzo[d]thiazole (4b) displayed an inhibitory effect on the proliferation of pancreatic cancer cell lines in a dose-dependent manner. Gemcitabine is generally used in therapy of pancreatic, bladder, breast, ovarian and non-small cell lung (26-30) cancer. Both compounds 4a and 4b demonstrated potent cytotoxicity towards PANC-1 cells, with IC_{50} of 27±0.24 μM and 35±0.51 μM, respectively. Gemcitabine, which was used as positive control, had an IC_{50} of 52±0.72 μM. We demonstrated that benzothiazole compounds had much

Figure 2. Effects of compound 4a (A) and 4b (B) on the viability of PANC-1 cells as determined by MTT assay. PANC-1 cells were treated with different concentrations of compounds 4a and 4b for 48 h. The values are the mean±SD presented as a percentage that of the untreated control. Significantly different at **p<0.01 and ***p<0.001 versus the control group.

Figure 3. Induction of apoptosis by compounds 4a (A) and 4b (B) in PANC-1 cells. PANC-1 cells were treated with different concentrations of synthesized benzothiazole compounds for 48 h. After 48 h of treatment, cell death detection ELISA was used to determine the apoptotic cell death. The values are expressed as mean±SD relative to the untreated control. Significantly different at *p<0.05, **p<0.01 and ***p<0.001 versus the control group.
Figure 4. Effect of compound 4a on superoxide dismutase (SOD) activity (A), glutathione peroxidase (GPx) activity (B) and total antioxidant capacity (TAC) (C) in PANC-1 cells. The values are expressed as means±SD. Significantly different at *p<0.05, **p<0.01 and ***p<0.001 versus the (untreated) control group.

Figure 5. Effect of compound 4b on superoxide dismutase (SOD) activity (A), glutathione peroxidase (GPx) activity (B), and total antioxidant capacity (TAC) (C) in PANC-1 cells. The values are expressed as means±SD. Significantly different at *p<0.05 and **p<0.01 versus the (untreated) control group.
higher cytotoxicity to the PANC-1 pancreatic cancer cell line in comparison with gemcitabine. In addition, the present study suggests that the inhibition of cell viability involves apoptosis observed in using cell death detection ELISA. Experimental results showed a concentration-dependent increase in apoptosis. Therefore, benzothiazole derivative compounds might act as potential antitumor compounds that are worth studying, particularly in relation to their effect on mechanisms of apoptosis and apoptosis signaling pathway.

Proteins are prone to alteration and damage by oxidative stress and these actions lead to different pathological conditions. Oxidative stress may be an early event in the activation of the apoptotic machinery (31). ROS at high concentrations are cytotoxic to pancreatic cancer cells. Therefore, ROS-mediated DNA damage abrogates malignant conversion of cells and tumorigenesis. Therapeutic attempts enhance pro-oxidant formation could preferably destroy pancreatic tumor cells through cellular oxidative stress. The ability of benzothiazole derivative compounds to increase cellular levels of ROS may be connected with their capacity to selectively target tumor cells (32, 33).

Available studies suggest that in many cancer cell lines, SOD activity was significantly higher in than healthy control groups regardless of tumor localization. According to our data, SOD activity was higher in untreated PANC-1 cells when compared to results obtained from PANC-1 cells treated with synthesized benzothiazole compounds. Rodrigues et al. evaluated anticancer effect of a new benzothiazole compound by investigating its antioxidant effects on breast cancer cells. Their results demonstrated that cancer cells were able to relatively recover from the cytotoxic effects of this compound. This compound caused significant inhibition of GPx and SOD activity (34). The results of the current study showed that glutathione-dependent enzyme (GPx) activities were significantly higher in untreated PANC-1 cells than in other groups. Likhar et al. designed and synthesized a series of 2-aryl substituted benzothiazole with various substituted benzoic acids. The compounds showed significant radical-scavenging capacity because of the presence of electron-donating substituents (35). These findings indicate that benzothiazole derivatives act as a defence mechanism by preventing the formation of excess free radicals. In our study, TAC were assessed in all groups an indicator of oxidative stress. Examination of TAC may be more functional instead of individual determination of oxidants and antioxidants (19). We found a significant difference between untreated PANC-1 cells and those treated with benzothiazole compounds in terms of mean TAC.

Inclusion of antioxidant drugs in the complex therapy of oncological patients may be important for control of oxidant–antioxidant imbalance. Thus, in this study we investigated applicability of benzothiazole compounds for correction of metabolic impairments through oxidative stress in PANC-1 cells. There is increased production of ROS by tumor cells and also induction of antioxidant systems (36). Synthesized compounds have been a primary source from which various apoptosis-inducing agents are obtained. As reported by various scientific studies, synthesized compounds may be effective in cancer inhibition or therapy (23, 37, 38). These studies demonstrated that bioactive compounds cause apoptosis of tumor cells (20, 39). In this regard, benzothiazole derivatives are well-recognized compounds used for the treatment and prevention of various tumors (40).

In conclusion, benzothiazole derivative compounds exhibited cytotoxic and apoptosis effects by regulating cell viability, antioxidant system in PANC-1 cancer cells. With further research, benzothiazole analogs may prove to be potent in assisting the treatment of tumor and improvement of anticancer drugs. The findings of this study demonstrated that the compound may be promising as an anticancer agent. However, further and larger in vitro and in vivo studies are needed to confirm these data and to establish fully the effect of synthesized benzothiazole compounds.

Conflicts of Interest

The Authors state no conflicts of interest exist in regard to this study.

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