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Research Article

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Synthesis and evaluation of new benzoxazole derivatives as potential antiglioma agents

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Received: 29 December 2017 / Revised: 05 March 2018 / Accepted: 11 March 2018

ABSTRACT: Gliomas account for the majority of human brain tumors and the incidence of gliomas is expected to rise in upcoming years and therefore extensive efforts have been devoted to the discovery of potent antiglioma agents. Due to the importance of benzoxazoles for anticancer drug discovery, herein new benzoxazole-based hydrazone derivatives (**3a-g**) were designed and synthesized. The cytotoxic effects of the compounds on C6 rat glioma and NIH/3T3 mouse embryonic fibroblast cell lines were investigated using MTT assay. The apoptotic effects of the most selective anticancer agent were analyzed based on Annexin V-PI binding capacities in flow cytometry. The compounds were also investigated for their acetylcholinesterase (AChE) inhibitory effects using a modification of Ellman's spectrophotometric method. In order to evaluate the compliance of the compounds to Lipinski's rule of five, their physicochemical parameters were determined using Molinspiration software. *N*'-([1,1'-Biphenyl]-4-ylmethylene)-2-[(5-fluorobenzoxazol-2-yl)thio]acetohydrazide (**3g**) was found to be more effective on C6 cell line (IC_{50}= 4.30±0.28 µg/mL) than mitoxantrone (IC_{50}= 4.56±1.24 µg/mL). The high SI value of compound **3g** indicated that its antiglioma activity was selective. This compound caused late apoptosis in a dose dependent manner. Compound **3g** was also found to be the most effective AChE inhibitor in this series. According to *in silico* studies, compound **3g** only violated one parameter of Lipinski's rule of five. On the basis of Lipinski's rule, the compound was expected to have reasonable oral bioavailability. According to *in vitro* and *in silico* studies, compound **3g** stands out as a promising antiglioma agent for further studies.

KEYWORDS: Acetylcholinesterase; apoptosis; benzoxazole; glioma; hydrazone.

1. INTRODUCTION

Gliomas are the most common primary malignant brain tumors and the incidence of gliomas is expected to rise in upcoming years. A common approach for the treatment of glioma involves surgery, radiotherapy and chemotherapy. However, there is no 100% curative therapy available for glioma patients and survival rates related to the most malignant types of gliomas are very low. Recurrences are also frequent since conventional therapies do not take into account the unique molecular features of different subtypes of glioma [1-3].

Efficacy of chemotherapy is limited due to poor drug delivery to the brain tumor and the correspondingly limited therapeutic response caused by partially intact blood-brain barrier (BBB) and blood-tumor barrier (BTB). Efficacy of anticancer agents is also reduced by the inherent chemoresistance of brain endothelial and glioma cells expressing the drug efflux protein, P-glycoprotein. As a result, extensive efforts have been devoted to the discovery of new antiglioma agents to improve drug delivery across the BBB/BTB and overcome drug resistance [1-3].

Benzoxazoles represent an important class of chemical entities in medicinal chemistry as building blocks for modulating a ligand's affinity and/or selectivity towards a particular target and therefore benzoxazole scaffold is often found in ligands targeting a plethora of receptors and enzymes. Benzoxazole derivatives have been reported to show a wide range of biological activities such as antibacterial, antifungal, antiviral, anticancer, and antioxidant activities [4-6]. In particular, recent studies have indicated that benzoxazole derivatives show potent antitumor activity against different cancer cell lines [7-12] through the inhibition of

How to cite this article: Altintop MD, Akalin Ciftci G, Temel HE. Synthesis and evaluation of new benzoxazole derivatives as potential antiglioma agents. Marmara Pharm J. 2018; 22 (4): 547-558.

https://doi.org/10.12991/jrp.2018.97
ISSN: 1309-0801

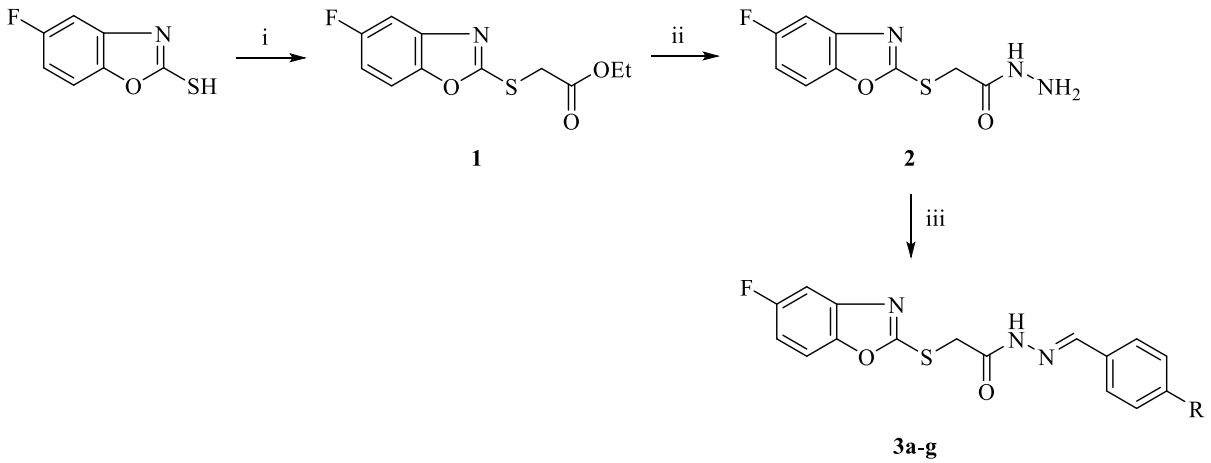
Aurora B kinase [8], cyclooxygenase-2 (COX-2) [9,10], topoisomerase II [11] and the induction of Caspase-3 dependent apoptosis [12].

Hydrazides-hydrazones have also attracted a great deal of interest as an important class of lead compounds for the development of new chemical entities to treat various diseases due to their unique structural features and diverse biological activities [13-15]. In particular, hydrazone moiety plays a pivotal role in anticancer drug development. Hydrazone derivatives have been reported to show potent antitumor activity against various cancer cell lines such as U-373 MG human glioblastoma, MCF-7 human breast adenocarcinoma, A549 human lung carcinoma, SK-OV-3 human ovary carcinoma, SK-MEL-2 human melanoma, HCT15 human colon carcinoma, MIA PaCa-2 human pancreas carcinoma, and HepG2 human hepatocellular carcinoma cell lines [14-20].

Prompted by the aforementioned findings, herein we designed, synthesized new benzoxazole-based hydrazone derivatives and tested their *in vitro* cytotoxic effects on C6 rat glioma and NIH/3T3 mouse embryonic fibroblast cell lines followed by flow cytometry-based apoptosis detection in C6 cell line. The compounds were also investigated for their acetylcholinesterase (AChE) inhibitory effects. A computational study for the prediction of absorption, distribution, metabolism and excretion (ADME) properties of all compounds was performed.

2. RESULTS AND DISCUSSION

The synthesis of new hydrazone derivatives (**3a-g**) was carried out according to the steps shown in Figure 1. In the initial step, ethyl 2-[(5-fluorobenzoxazol-2-yl)thio]acetate (**1**) was synthesized *via* the reaction of 5-fluorobenzoxazole-2-thiol with ethyl chloroacetate in the presence of potassium carbonate. The treatment of the ester (**1**) with hydrazine hydrate afforded the corresponding hydrazide (**2**). Finally, the nucleophilic addition-elimination reaction of the hydrazide (**2**) with aromatic aldehydes gave the target compounds (**3a-g**).



Compound	R
3a	H
3b	NO_2
3c	CN
3d	F
3e	Cl
3f	Br
3g	Phenyl

Figure 1. The synthetic route for the preparation of compounds **3a-g**. Reagents and conditions: (i) $ClCH_2COOEt$, K_2CO_3, acetone, reflux, 10 h; (ii) $NH_2NH_2.H_2O$, ethanol, rt, 3 h; (iii) Aromatic aldehyde, ethanol, reflux, 6 h.

The structures of the compounds were confirmed by IR, ^1H NMR, ^{13}C NMR, mass spectral data, and elemental analysis. In the IR spectrum of compound **2**, the C=O stretching vibrations gave rise to a band at 1641.42 cm^{-1}. The N-H stretching bands were observed in the region 3298-3203 cm^{-1}. The stretching bands for aromatic and aliphatic C-H groups occurred at 3111-3043 cm^{-1} and 2995-2939 cm^{-1}, respectively. N-H bending,

C=N, C=C stretching bands were observed in the region 1625-1462 cm^{-1}. In the IR spectra of compounds **3a-g**, a strong, characteristic band in the region 1687-1658 cm^{-1} due to the C=O stretching vibration was observed. The N-H stretching bands occurred at 3253-3184 cm^{-1}. The aromatic and aliphatic C-H stretching vibrations gave rise to bands at 3142-3030 cm^{-1} and 2980-2846 cm^{-1}, respectively. C=N, C=C stretching and N-H bending vibrations were observed in the region 1616-1463 cm^{-1}. In the IR spectrum of cyano-substituted compound **3c**, the C≡N stretching band occurred at 2229.71 cm^{-1}.

In the ^{1}H NMR spectrum of compound **2**, the signals due to the NH_2 and NH protons of the hydrazide group appeared at 4.35 and 9.43 ppm, respectively. The signal due to the S-CH_2 protons was observed at 4.08 ppm as a singlet. In the ^{1}H NMR spectra of compounds **3a-g**, the signal due to the hydrazone proton appeared in the region 11-12 ppm as a singlet. The signals due to the S-CH_2 protons were observed in the region 4.28-4.73 ppm. The signal belonging to CH=N proton appeared in the region 8.01-8.33 ppm. In the ^{1}H NMR spectra of compounds **3a-g**, CH=N and S-CH_2 protons gave rise to two singlet peaks in accordance with the presence of the *E* and *Z* isomers [21,22].

In the ^{13}C NMR spectra of all compounds, the signal due to the S-CH_2 carbon was observed in the region 34-35 ppm. The signal due to the C=O carbon was observed at 165-169 ppm. In the ^{13}C NMR spectra of compounds **3a-g**, the signal due to the hydrazone carbon was observed at 144-147 ppm. In the ^{13}C NMR spectrum of cyano-substituted compound **3c**, the signal due to the C≡N carbon appeared at 119.10 ppm. HRMS spectral data and elemental analysis were in agreement with the proposed structures of the compounds.

MTT assay was carried out to determine the anticancer effects of the compounds on C6 rat glioma cell line (Table 1). Biphenyl-substituted compound **3g** was found to be the most potent anticancer agent against C6 cell line with an IC_{50} value of 4.30±0.28 µg/mL when compared with mitoxantrone (IC_{50}= 4.56±1.24 µg/mL). This outcome indicated that biphenyl group significantly enhanced anticancer activity against C6 cell line. The increased anticancer activity can be attributed to the increased lipophilicity of the compound due to the presence of biphenyl moiety.

Toxicity to host cells is an important characteristic to assess the safety of drug candidates early in the drug discovery process. The cytotoxic effects of the compounds on NIH/3T3 mouse embryonic fibroblast cell line were investigated to evaluate whether the compounds were toxic or non-toxic to healthy cells. The selectivity index (SI) values of the compounds were also determined to compare the selectivity of the compounds (Table 1). The high SI value of compound **3g** pointed out that the antiglioma activity of compound **3g** was selective. Due to its notable and selective antitumor effect on C6 cells, compound **3g** was chosen for further studies.

Table 1. IC_{50} values of the compounds against C6 and NIH/3T3 cells for 24 h

Compound	IC_{50} values (µg/mL)		SI value*
	C6 cell line	NIH/3T3 cell line	
2	>500	>500	-
3a	>500	>500	-
3b	46.00±6.92	51.67±2.89	1.12
3c	>500	>500	-
3d	>500	>500	-
3e	>500	>500	-
3f	>500	>500	-
3g	4.30±0.28	>500	>116.28
Mitoxantrone	4.56±1.24	ND	-

ND: Not determined.
* SI= IC_{50} for normal cell line / IC_{50} for cancerous cell line.

After 24 h incubation period, the apoptotic effects of compound **3g** were analyzed based on Annexin V-PI binding capacities in flow cytometry (Table 2, Figure 2). Following flow cytometric analyses, the early and late apoptotic effects of compound **3g** (for $IC_{50}/2$ and IC_{50} doses) on C6 cell line were determined as 9.3% and 8.7%, respectively. According to these findings, the early apoptotic effects of this compound did not increase in a dose dependent manner, whereas the late apoptotic effects increased in a dose dependent manner.

Table 2. Percents of typical quadrant analysis of Annexin V FITC/Propidium iodide (PI) flow cytometry of C6 cells treated with compound **3g** and mitoxantrone at $IC_{50}/2$ and IC_{50} concentrations

Groups	Early apoptotic cells%	Late apoptotic cells%	Viable cells%
Control (untreated)	2.5	3.2	92.8
Compound **3g** $IC_{50}/2$ dose treated cells	2.7	6.6	70.9
Compound **3g** IC_{50} dose treated cells	0.8	7.9	87.3
Mitoxantrone $IC_{50}/2$ dose treated cells	2.9	7.9	43.4
Mitoxantrone IC_{50} dose treated cells	1.6	8.0	40.5

C6 cells were cultured for 24 h in medium with compound **3g** and mitoxantrone at $IC_{50}/2$ and IC_{50} concentrations. At least 10.000 cells were analyzed per sample, and quadrant analysis was performed.

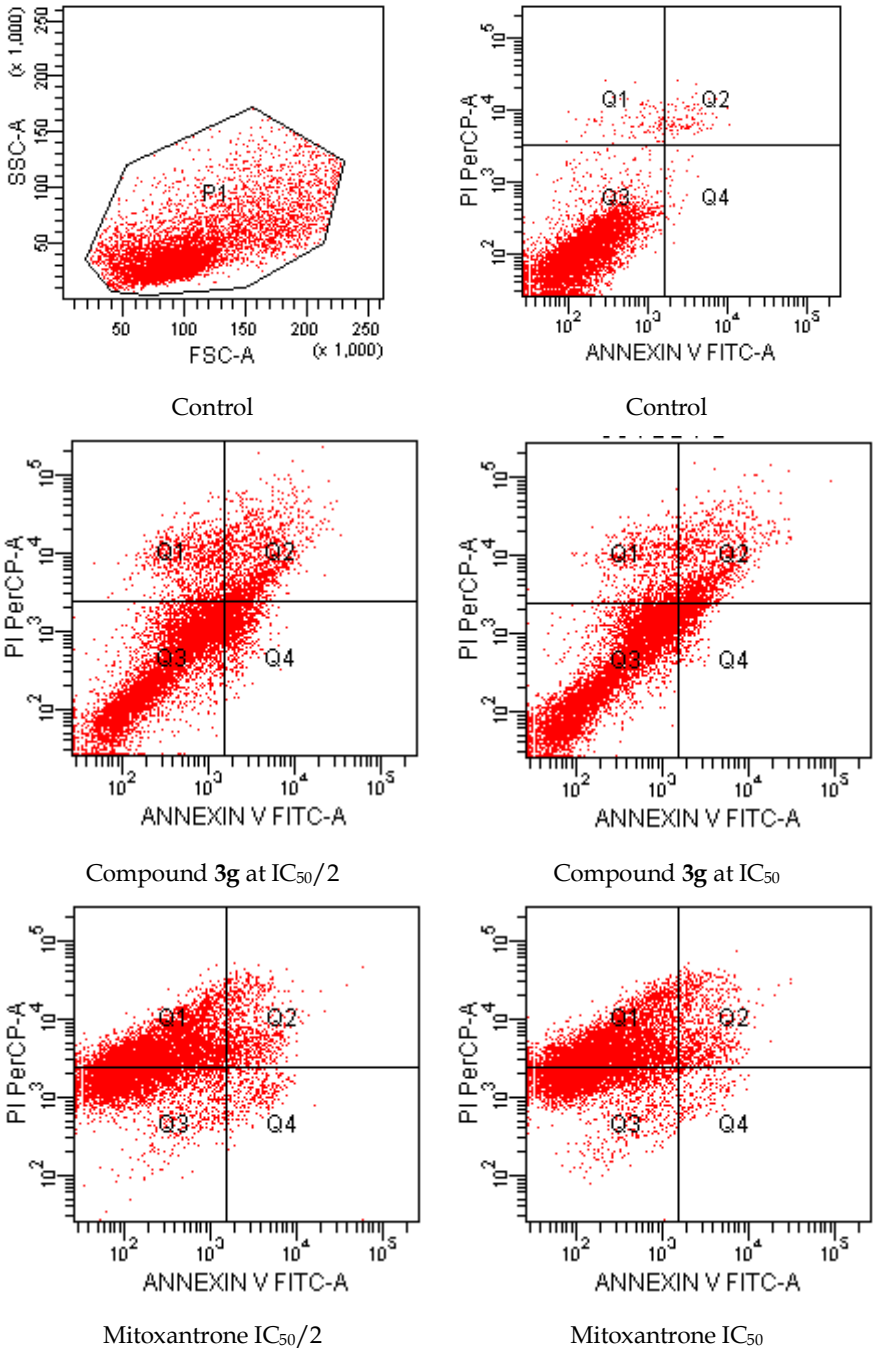


Figure 2. Flow cytometric analysis of C6 cells treated with compound **3g** and mitoxantrone at IC_{50} and $IC_{50}/2$ concentrations. C6 cells were cultured for 24 h in medium with compound **3g** and mitoxantrone at $IC_{50}/2$ and IC_{50} concentrations. At least 10,000 cells were analyzed per sample, and quadrant analysis was performed.

The inhibitory effects of the compounds on AChE were also determined by a modification of Ellman's spectrophotometric method (Table 3). Galantamine was used as a positive control. According to the results, compounds **3a-g** showed less AChE inhibitory activity than galantamine. Compounds **2**, **3a**, **3b**, **3c**, **3d**, **3f** and **3g** showed less than 50% AChE inhibition at 80 µg/mL, whereas compound **3e** did not show any inhibitory activity against AChE. Compound **3g** was identified as the most potent AChE inhibitor (45.98±3.13%) in this series. This outcome indicated that biphenyl moiety enhanced AChE inhibitory activity. The increased activity can be attributed to its high lipophilicity due to the presence of biphenyl moiety.

Table 3. The inhibitory effects of the compounds on AChE

Compound	AChE% inhibition (80 µg/mL)
2	19.77±2.29
3a	27.41±1.88
3b	28.48±2.71
3c	18.08±3.06
3d	19.93±3.20
3e	---
3f	10.91±0.76
3g	45.98±3.13
Galantamine	(0.45±0.04)[a]

---: No inhibition
[a] IC_{50} value (µg/mL)

In order to evaluate the compliance of the compounds to Lipinski's rule of five, Molinspiration software was used to determine their physicochemical parameters (log P, TPSA, nrotb, molecular weight, number of hydrogen bond donors and acceptors, molecular volume) [23]. According to Lipinski's rule of five, most "drug like" molecules have log P ≤ 5, molecular weight ≤ 500, number of hydrogen bond acceptors ≤ 10, and number of hydrogen bond donors ≤ 5 and compounds violating more than one of these rules may have bioavailability problems [23-26]. According to *in silico* studies, compound **3g** only violated one parameter of Lipinski's rule of five, whereas other compounds did not violate Lipinski's rule (Table 4). On the basis of Lipinski's rule of five, they were expected to have good oral bioavailability.

Table 4. The predicted pharmacokinetic parameters of the compounds

Compound	Molecular properties [a]							
	MW	logP	TPSA	nrotb	HBA	HBD	Volume	Violations
2	241.25	0.25	81.15	3	5	3	187.98	0
3a	329.36	3.66	67.49	5	5	1	271.36	0
3b	374.35	3.62	113.32	6	8	1	294.70	0
3c	354.37	3.42	91.29	5	6	1	288.22	0
3d	347.35	3.83	67.49	5	5	1	276.30	0
3e	363.80	4.34	67.49	5	5	1	284.90	0
3f	408.25	4.47	67.49	5	5	1	289.25	0
3g	405.45	5.46	67.49	6	5	1	342.77	1

[a] Molecular properties were calculated using Molinspiration software. MW: Molecular weight; logP: The logarithm of octanol/water partition coefficient, TPSA: Topological polar surface area; nrotb: Number of rotatable bonds, HBA: Number of hydrogen bond acceptors, HBD: Number of hydrogen bond donors.

3. CONCLUSION

In the present paper, new benzoxazole-based hydrazone derivatives were synthesized and investigated for their cytotoxic effects on C6 rat glioma, and NIH/3T3 mouse embryonic fibroblast cell lines and AChE inhibitory effects. Moreover, the compliance of the compounds to the Lipinski's rule of five was evaluated.

Biphenyl-substituted compound **3g** was identified as the most promising anticancer agent against C6 cell line due to its selective and notable anticancer activity (IC_{50}= 4.30±0.28 µg/mL) when compared with mitoxantrone (IC_{50}= 4.56±1.24 µg/mL). The apoptotic effects of compound **3g** were also investigated. This compound caused late apoptosis in a dose dependent manner. According to the *in vitro* and *in silico* studies, compound **3g** stands out as a promising orally bioavailable anticancer drug candidate for further *in vitro* and *in vivo* studies.

4. MATERIALS AND METHODS

4.1. Chemistry

All reagents were purchased from commercial suppliers and were used without further purification. The melting points (M.p.) of the compounds were determined on a MP90 digital melting point apparatus (Mettler Toledo, Ohio, USA) and are uncorrected. IR spectra were recorded on an IRPrestige-21 Fourier Transform Infrared spectrophotometer (Shimadzu, Tokyo, Japan). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker spectrometer (Bruker, Billerica, MA, USA). Chemical shifts were reported in parts per million (ppm) and the coupling constants (*J*) were expressed in Hertz (Hz). Mass spectra were recorded on a Shimadzu LCMS-IT-TOF system (Shimadzu, Kyoto, Japan). Elemental analyses (C, H, N) were performed on a Perkin Elmer EAL 240 elemental analyzer (Perkin-Elmer, Norwalk, CT, USA). Thin Layer Chromatography (TLC) was performed on TLC Silica gel 60 F254 aluminium sheets (Merck, Darmstadt, Germany) to check the purity of the compounds.

General procedure for the synthesis of the compounds

*Ethyl 2-[(5-fluorobenzoxazol-2-yl)thio]acetate (**1**)*

A mixture of 5-fluorobenzoxazole-2-thiol (0.05 mol) and ethyl chloroacetate (0.05 mol) in the presence of potassium carbonate (0.05 mol) in acetone was refluxed for 10 h. The reaction mixture was cooled, filtered and the crude product was solved in water and then extracted with ether [22].

*2-[(5-Fluorobenzoxazol-2-yl)thio]acetohydrazide (**2**)*

A mixture of the ester (**1**) (0.05 mol) and hydrazine hydrate (0.1 mol) in ethanol was stirred at room temperature for 3 h and then filtered [22].

M.p. 174.6 °C. Yield 88%.

IR ν_{max} (cm^{-1}): 3298.28, 3203.76 (N-H stretching), 3111.18, 3089.96, 3043.67 (Aromatic C-H stretching), 2995.45, 2939.52 (Aliphatic C-H stretching), 1641.42 (C=O stretching), 1625.99, 1537.27, 1494.83, 1473.62, 1462.04 (N-H bending, C=N and C=C stretching), 1435.04, 1408.04 (C-H bending), 1338.60, 1269.16, 1251.80, 1238.30, 1217.08, 1178.51, 1139.93, 1128.36, 1001.06 (C-N, C-O stretching and aromatic C-H in plane bending), 956.69, 935.48, 854.47, 831.32, 804.32, 771.53, 727.16, 678.94 (Aromatic C-H out of plane bending and C-S stretching).

^{1}H NMR (300 MHz, DMSO-d_6): 4.08 (s, 2H, S-CH_2), 4.35 (s, 2H, NH_2), 7.13-7.20 (m, 1H, benzoxazole), 7.50 (dd, *J*= 2.61 Hz, *J*= 8.76 Hz, 1H, benzoxazole), 7.66 (dd, *J*= 4.35 Hz, *J*= 8.91 Hz, 1H, benzoxazole), 9.43 (s, 1H, N-H).

^{13}C NMR (75 MHz, DMSO-d_6): 34.43 (CH_2, S-CH_2), 105.42 (CH, d, *J*= 26.25 Hz, benzoxazole), 111.37 (CH, d, *J*= 10.5 Hz, benzoxazole), 111.92 (CH, d, *J*= 26.25 Hz, benzoxazole), 142.58 (C, d, *J*= 13.5 Hz, benzoxazole), 148.27 (C, benzoxazole), 158.32 (C, benzoxazole), 161.47 (C, benzoxazole), 165.96 (C, C=O).

HRMS (ESI) (*m/z*): $[M+H]^+$ calcd. for $C_9H_8FN_3O_2S$: 242.0394, found: 242.0387.

For $C_9H_8FN_3O_2S$ Calculated: C, 44.81; H, 3.34; N, 17.42. Found: C, 44.78; H, 3.36; N, 17.43.

*N'-Benzylidene-2-[(5-fluorobenzoxazol-2-yl)thio]acetohydrazide derivatives (**3a-g**)*

A mixture of the hydrazide (**2**) (0.01 mol) and appropriate aldehyde (0.01 mol) was refluxed in ethanol for 6 h, filtered and crystallized from ethanol [22].

*N'-Benzylidene-2-[(5-fluorobenzoxazol-2-yl)thio]acetohydrazide (**3a**)*

M.p. 190.5 °C. Yield 82%.

IR ν_{max} (cm^{-1}): 3184.48 (N-H stretching), 3099.61 (Aromatic C-H stretching), 2976.16, 2870.08 (Aliphatic C-H stretching), 1672.28 (C=O stretching), 1614.42, 1490.97, 1473.62, 1463.97 (N-H bending, C=N and C=C stretching), 1448.54, 1435.04, 1388.75, 1367.53, 1352.10 (C-H bending), 1315.45, 1234.44, 1203.58, 1130.29, 1074.35 (C-N, C-O stretching and aromatic C-H in plane bending), 952.84, 927.76, 898.83, 885.33, 860.25, 829.39, 798.53, 783.10, 750.31, 690.52 (Aromatic C-H out of plane bending and C-S stretching).

^{1}H NMR (300 MHz, DMSO-d_6): 4.29 and 4.69 (2s, 2H, S-CH_2), 7.16 (t, *J*= 8.46 Hz, 1H, Ar-H), 7.43 (s, 1H, Ar-H), 7.52 (d, *J*= 8.55 Hz, 3H, Ar-H), 7.69 (brs, 3H, Ar-H), 8.05 and 8.22 (2s, 1H, CH=N), 11.79 (s, 1H, N-H).

^{13}C NMR (75 MHz, DMSO-d_6): 35.28 (CH_2, S-CH_2), 105.47 (CH, d, *J*= 26.25 Hz, aromatic), 111.33 (CH, d, *J*= 10.5 Hz, aromatic), 111.85 (CH, d, *J*= 26.25 Hz, aromatic), 127.51 (2CH, d, *J*= 16.5 Hz, aromatic), 129.29 (2CH, aromatic), 130.60 (CH, d, *J*= 10.5 Hz, aromatic), 134.39 (C, d, *J*= 8.25 Hz, aromatic), 142.64 (C, d, *J*= 12.75 Hz, aromatic), 144.62 (CH, CH=N), 147.76 (C, aromatic), 158.32 (C, aromatic), 161.47 (C, aromatic), 168.34 (C, C=O).

HRMS (ESI) (*m/z*): $[M+H]^+$ calcd. for $C_{16}H_{12}FN_3O_2S$: 330.0707, found: 330.0693.

For $C_{16}H_{12}FN_3O_2S$ Calculated: C, 58.35; H, 3.67; N, 12.76. Found: C, 58.31; H, 3.68; N, 12.79.

*2-[(5-Fluorobenzoxazol-2-yl)thio]-N'-(4-nitrobenzylidene)acetohydrazide (**3b**)*

M.p. 237.3 °C. Yield 91%.

IR ν_{max} (cm^{-1}): 3197.98 (N-H stretching), 3076.46 (Aromatic C-H stretching), 2953.02, 2846.93 (Aliphatic C-H stretching), 1678.07 (C=O stretching), 1614.42, 1585.49, 1519.91, 1485.19, 1469.76 (N-H bending, NO_2, C=N and C=C stretching), 1404.18, 1381.03 (C-H bending), 1338.60, 1249.87, 1234.44, 1201.65, 1138.00, 1105.21 (C-N, C-O stretching and aromatic C-H in plane bending), 952.84, 927.76, 898.83, 875.68, 852.54, 833.25, 806.25, 771.53, 748.38, 690.52 (Aromatic C-H out of plane bending and C-S stretching).

^{1}H NMR (300 MHz, DMSO-d_6): 4.32 and 4.73 (2s, 2H, S-CH_2), 7.17 (t, *J*= 9.0 Hz, *J*= 9.21 Hz, 1H, Ar-H), 7.53 (d, *J*= 8.61 Hz, 1H, Ar-H), 7.67-7.69 (m, 1H, Ar-H), 7.98 (d, *J*= 7.68 Hz, 2H, Ar-H), 8.15 and 8.33 (s, 1H, CH=N), 8.28 (d, *J*= 8.13 Hz, 2H, Ar-H), 12.09 (s, 1H, N-H).

^{13}C NMR (75 MHz, DMSO-d_6): 35.14 (CH_2, S-CH_2), 105.50 (CH, d, *J*= 25.5 Hz, aromatic), 111.39 (CH, d, *J*= 10.5 Hz, aromatic), 111.92 (CH, d, *J*= 26.25 Hz, aromatic), 124.51 (2CH, aromatic), 128.38 (2CH, aromatic), 140.63 (C, aromatic), 142.27 (C, aromatic), 145.34 (CH, CH=N), 148.29 (C, aromatic), 158.33 (C, aromatic), 161.49 (C, aromatic), 163.72 (C, aromatic), 168.77 (C, C=O).

HRMS (ESI) (*m/z*): $[M+H]^+$ calcd. for $C_{16}H_{11}FN_4O_4S$: 375.0558, found: 375.0542.

For $C_{16}H_{11}FN_4O_4S$ Calculated: C, 51.34; H, 2.96; N, 14.97. Found: C, 51.37; H, 2.94; N, 14.96.

*N'-(4-Cyanobenzylidene)-2-[(5-fluorobenzoxazol-2-yl)thio]acetohydrazide (**3c**)*

M.p. 253.6 °C. Yield 90%.

IR ν_{max} (cm^{-1}): 3224.98 (N-H stretching), 3140.11, 3097.68, 3057.17 (Aromatic C-H stretching), 2978.09, 2941.44 (Aliphatic C-H stretching), 2229.71 (C≡N stretching), 1678.07 (C=O stretching), 1598.99, 1500.62, 1475.54, 1467.83 (N-H bending, C=N and C=C stretching), 1442.75, 1431.18, 1375.25 (C-H bending), 1346.31, 1309.67, 1219.01, 1201.65, 1130.29, 1105.21 (C-N, C-O stretching and aromatic C-H in plane bending), 954.76, 941.26, 881.47, 854.47, 844.82, 823.60, 785.03, 769.60, 677.01 (Aromatic C-H out of plane bending and C-S stretching).

^{1}H NMR (300 MHz, DMSO-d_6): 4.31 and 4.70 (2s, 2H, S-CH_2), 7.15 (t, *J*= 8.82 Hz, *J*= 8.91 Hz, 1H, Ar-H), 7.50 (d, *J*= 8.31 Hz, 1H, Ar-H), 7.64-7.66 (m, 1H, Ar-H), 7.88 (s, 4H, Ar-H), 8.08 and 8.27 (2s, 1H, CH=N), 12.03 (s, 1H, N-H).

^{13}C NMR (75 MHz, DMSO-d_6): 35.14 (CH_2, S-CH_2), 105.46 (CH, d, *J*= 26.25 Hz, aromatic), 111.33 (CH, d, *J*= 9.75 Hz, aromatic), 111.87 (CH, d, *J*= 26.25 Hz, aromatic), 112.33 (C, aromatic), 119.10 (C, C≡N), 127.97 (2CH, aromatic), 133.16 (2CH, aromatic), 138.77 (C, aromatic), 142.68 (C, aromatic), 145.83 (CH, CH=N), 148.28 (C, aromatic), 158.31 (C, aromatic), 161.47 (C, aromatic), 168.70 (C, C=O).

HRMS (ESI) (*m/z*): $[M+H]^+$ calcd. for $C_{17}H_{11}FN_4O_2S$: 355.0660, found: 355.0643.

For $C_{17}H_{11}FN_4O_2S$ Calculated: C, 57.62; H, 3.13; N, 15.81. Found: C, 57.65; H, 3.11; N, 15.80.

*2-[(5-Fluorobenzoxazol-2-yl)thio]-N'-(4-fluorobenzylidene)acetohydrazide (**3d**)*

M.p. 204.7 °C. Yield 84%.

IR ν_{max} (cm^{-1}): 3197.98 (N-H stretching), 3120.82, 3078.39, 3061.03 (Aromatic C-H stretching), 2980.02, 2960.73 (Aliphatic C-H stretching), 1674.21 (C=O stretching), 1616.35, 1600.92, 1498.69, 1479.40, 1469.76 (N-H bending, C=N and C=C stretching), 1435.04, 1381.03, 1357.89 (C-H bending), 1340.53, 1301.95, 1267.23, 1230.58, 1217.08, 1130.29, 1103.28 (C-N, C-O stretching and aromatic C-H in plane bending), 954.76, 931.62, 881.47, 860.25, 848.68, 812.03, 790.81, 769.60, 717.52, 677.01 (Aromatic C-H out of plane bending and C-S stretching).

^{1}H NMR (300 MHz, DMSO-d_6): 4.29 and 4.67 (2s, 2H, S-CH_2), 7.14 (t, *J*= 7.47 Hz, *J*= 8.88 Hz, 1H, Ar-H), 7.26 (t, *J*= 8.37 Hz, *J*= 8.46 Hz, 2H, Ar-H), 7.49 (d, *J*= 8.46 Hz, 1H, Ar-H), 7.63-7.65 (m, 1H, Ar-H), 7.76 (s, 2H, Ar-H), 8.04 and 8.22 (2s, 1H, CH=N), 11.80 (s, 1H, N-H).

^{13}C NMR (75 MHz, DMSO-d_6): 35.24 (CH_2, S-CH_2), 105.42 (CH, d, *J*= 26.25 Hz, aromatic), 111.27 (CH, d, *J*= 9.75 Hz, aromatic), 111.80 (CH, d, *J*= 26.25 Hz, aromatic), 116.31 (2CH, d, *J*= 21.75 Hz, aromatic), 129.56 (CH, d, *J*= 8.25 Hz, aromatic), 129.80 (CH, d, *J*= 8.25 Hz, aromatic), 130.93 (C, aromatic), 143.47 (C, aromatic), 146.66 (CH, CH=N), 148.28 (C, aromatic), 158.30 (C, aromatic), 161.46 (C, aromatic), 165.17 (C, aromatic), 168.33 (C, C=O).

HRMS (ESI) (*m/z*): $[M+H]^+$ calcd. for $C_{16}H_{11}F_2N_3O_2S$: 348.0613, found: 348.0597.

For $C_{16}H_{11}F_2N_3O_2S$ Calculated: C, 55.33; H, 3.19; N, 12.10. Found: C, 55.35; H, 3.21; N, 12.07.

*N'-(4-Chlorobenzylidene)-2-[(5-fluorobenzoxazol-2-yl)thio]acetohydrazide (**3e**)*

M.p. 208.9 °C. Yield 86%.

IR ν_{max} (cm^{-1}): 3230.77 (N-H stretching), 3138.18, 3076.46, 3057.17 (Aromatic C-H stretching), 2978.09 (Aliphatic C-H stretching), 1672.28 (C=O stretching), 1612.49, 1502.55, 1467.83 (N-H bending, C=N and C=C stretching), 1433.11, 1377.17, 1355.96 (C-H bending), 1338.60, 1303.88, 1219.01, 1203.58, 1128.36, 1103.28, 1087.85, 1014.56 (C-N, C-O stretching and aromatic C-H in plane bending), 952.84, 931.62, 881.47, 860.25, 839.03, 810.10, 783.10, 767.67, 713.66, 677.01 (Aromatic C-H out of plane bending and C-S stretching).

^{1}H NMR (300 MHz, DMSO-d_6): 4.28 and 4.68 (2s, 2H, S-CH_2), 7.16 (t, *J*= 8.97 Hz, *J*= 9.06 Hz, 1H, Ar-H), 7.49 (d, *J*= 7.17 Hz, 3H, Ar-H), 7.65-7.68 (m, 1H, Ar-H), 7.73 (d, *J*= 8.04 Hz, 2H, Ar-H), 8.03 and 8.21 (2s, 1H, CH=N), 11.86 (s, 1H, N-H).

^{13}C NMR (75 MHz, DMSO-d_6): 35.20 (CH_2, S-CH_2), 105.46 (CH, d, *J*= 26.25 Hz, aromatic), 111.33 (CH, d, *J*= 10.5 Hz, aromatic), 111.85 (CH, d, *J*= 25.5 Hz, aromatic), 129.04 (2CH, aromatic), 129.36 (2CH, aromatic), 133.29 (C, aromatic), 134.96 (C, aromatic), 143.33 (C, aromatic), 146.47 (CH, CH=N), 148.29 (C, aromatic), 158.31 (C, aromatic), 161.47 (C, aromatic), 168.40 (C, C=O).

HRMS (ESI) (*m/z*): $[M+H]^+$ calcd. for $C_{16}H_{11}ClFN_3O_2S$: 364.0317, found: 364.0304.

For $C_{16}H_{11}ClFN_3O_2S$ Calculated: C, 52.83; H, 3.05; N, 11.55. Found: C, 52.79; H, 3.06; N, 11.58.

*N'-(4-Bromobenzylidene)-2-[(5-fluorobenzoxazol-2-yl)thio]acetohydrazide (**3f**)*

M.p. 194.4 °C. Yield 88%.

IR ν_{max} (cm^{-1}): 3246.20 (N-H stretching), 3142.04, 3076.46, 3055.24 (Aromatic C-H stretching), 2978.09 (Aliphatic C-H stretching), 1687.71 (C=O stretching), 1608.63, 1502.55, 1467.83 (N-H bending, C=N and C=C stretching), 1433.11, 1402.25, 1375.25, 1354.03 (C-H bending), 1336.67, 1303.88, 1217.08, 1203.58, 1126.43, 1101.35, 1070.49, 1010.70 (C-N, C-O stretching and aromatic C-H in plane bending), 952.84, 931.62, 881.47, 860.25, 835.18, 808.17, 781.17, 769.60, 707.88, 677.01 (Aromatic C-H out of plane bending and C-S stretching).

^{1}H NMR (300 MHz, DMSO-d_6): 4.29 and 4.67 (2s, 2H, S-CH_2), 7.14 (t, *J*= 8.19 Hz, *J*= 8.73 Hz, 1H, Ar-H), 7.49 (d, *J*= 8.43 Hz, 1H, Ar-H), 7.63-7.66 (m, 5H, Ar-H), 8.01 and 8.19 (2s, 1H, CH=N), 11.86 (s, 1H, N-H).

^{13}C NMR (75 MHz, DMSO-d_6): 35.23 (CH_2, S-CH_2), 105.44 (CH, d, *J*= 26.25 Hz, aromatic), 111.30 (CH, d, *J*= 9.75 Hz, aromatic), 111.83 (CH, d, *J*= 26.25 Hz, aromatic), 123.75 (C, aromatic), 129.24 (2CH, aromatic), 132.25 (2CH, aromatic), 133.61 (C, aromatic), 143.43 (C, aromatic), 146.56 (CH, CH=N), 148.28 (C, aromatic), 158.30 (C, aromatic), 161.46 (C, aromatic), 168.40 (C, C=O).

HRMS (ESI) (*m/z*): $[M+H]^+$ calcd. for $C_{16}H_{11}BrFN_3O_2S$: 407.9812, found: 407.9814.

For $C_{16}H_{11}BrFN_3O_2S$ Calculated: C, 47.07; H, 2.72; N, 10.29. Found: C, 47.07; H, 2.70; N, 10.31.

*N'-([1,1'-Biphenyl]-4-ylmethylene)-2-[(5-fluorobenzoxazol-2-yl)thio]acetohydrazide (**3g**)*

M.p. 215.8 °C. Yield 88%.

IR ν_{max} (cm^{-1}): 3253.91 (N-H stretching), 3059.10, 3030.17 (Aromatic C-H stretching), 2927.94 (Aliphatic C-H stretching), 1658.78 (C=O stretching), 1606.70, 1544.98, 1494.83, 1471.69 (N-H bending, C=N and C=C stretching), 1436.97, 1402.25, 1361.74 (C-H bending), 1311.59, 1238.30, 1193.94, 1136.07, 1130.29, 1064.71 (C-N, C-O stretching and aromatic C-H in plane bending), 991.41, 968.27, 956.69, 935.48, 887.26, 833.25, 813.96, 761.88, 736.81, 723.31, 713.66, 688.59, 675.09 (Aromatic C-H out of plane bending and C-S stretching).

^{1}H NMR (300 MHz, DMSO-d_6): 4.30 and 4.71 (2s, 2H, S-CH_2), 7.14-7.20 (m, 1H, Ar-H), 7.39-7.55 (m, 4H, Ar-H), 7.66-7.82 (m, 7H, Ar-H), 8.09 and 8.26 (2s, 1H, CH=N), 11.84 (s, 1H, N-H).

^{13}C NMR (75 MHz, DMSO-d_6): 35.29 (CH_2, S-CH_2), 105.50 (CH, d, *J*= 26.25 Hz, aromatic), 111.37 (CH, d, *J*= 10.5 Hz, aromatic), 111.88 (CH, d, *J*= 26.25 Hz, aromatic), 127.14 (2CH, aromatic), 127.51 (2CH, aromatic), 128.02 (2CH, aromatic), 128.38 (CH, aromatic), 129.50 (2CH, aromatic), 133.45 (C, aromatic), 139.75 (C, aromatic), 142.16 (2C, aromatic), 144.22 (CH, CH=N), 147.34 (C, aromatic), 158.33 (C, aromatic), 161.48 (C, aromatic), 168.35 (C, C=O).

HRMS (ESI) (*m/z*): $[M+H]^+$ calcd. for $C_{22}H_{16}FN_3O_2S$: 406.1020, found: 406.1010.

For $C_{22}H_{16}FN_3O_2S$ Calculated: C, 65.17; H, 3.98; N, 10.36. Found: C, 65.15; H, 3.99; N, 10.37.

4.2. Biochemistry

4.2.1. Cytotoxicity

Cell culture and drug treatment

C6 Rat glioma and NIH/3T3 mouse embryonic fibroblast cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Deisenhofen, Germany) supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland). All media were supplemented with 100 IU/mL penicillin-streptomycin (Gibco, Paisley, Scotland) and cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Exponentially growing cells were plated at $2x10^4$ cells/mL into 96-well microtiter tissue culture plates (Nunc, Denmark) and incubated for 24 h before the addition of the drugs (the optimum cell number for cytotoxicity assays was

determined in preliminary experiments). The stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO; Sigma Aldrich, Poole, UK) and further dilutions were made with fresh culture medium (the concentration of DMSO in the final culture medium was <0.1% which had no effect on the cell viability).

MTT assay

The level of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) reduction was quantified as previously described in the literature [27,28] with small modifications [29].

After 24 h of preincubation, the compounds and mitoxantrone (positive control) were added to give final concentration in the range 3.9-500 µg/mL and the cells were incubated for 24 h. At the end of this period, MTT was added to a final concentration of 0.5 mg/mL and the cells were incubated for 4 h at 37 °C. After the medium was removed, the formazan crystals formed by MTT metabolism were solubilized by addition of 200 µL DMSO to each well and absorbance was read at 540 nm with a microtiter plate spectrophotometer (Bio-Tek plate reader, Winooski, VT, USA). Every concentration was repeated in three wells. IC_{50} values were defined as the drug concentrations that reduced absorbance to 50% of control values.

Selectivity index (SI) values were calculated according to the formula [30] below:

SI= IC_{50} for normal cell line / IC_{50} for cancerous cell line

4.2.2. Flow cytometric analyses of apoptosis

After the cells were incubated with compound **3g** and mitoxantrone at $IC_{50}/2$ and IC_{50} concentrations, phosphatidylserine externalization, which indicates early apoptosis, was measured by FITC Annexin V apoptosis detection kit (BD Pharmingen, San Jose, CA, USA) on a BD FACSAria flow cytometer for 24 h. Annexin V staining protocol was applied according to the manufacturer's instructions (BD Pharmingen, San Jose, CA, USA). The cells were then briefly washed with cold phosphate buffer saline (PBS) and suspended in a binding buffer at a concentration of 1×10^6 cells/mL. Then, 100 µL of this solution containing 1×10^5 cells was transferred to a 5 mL test tube. After 5 µL of Annexin V and PI was added, the cells were incubated for 15 min at room temperature in the dark. Then 400 µL of 1x binding buffer was added to each tube and the cells were processed for data acquisition, and analyzed on a BD FACSAria flow cytometer using FACSDiva version 6.1.1 software (BD Biosciences, San Jose, CA, USA) [29].

4.2.3. AChE inhibitory activity

The AChE inhibitory effects of the compounds were determined by Ellman's method [31] with minor modifications (Electric eel AChE was used instead of bovine AChE and buffer was added 2.4 mL instead of 3 mL) [32]. The compounds were dissolved in DMSO and tested at final concentration range from 5 to 80 µg/mL. 20 µL of AChE (1 U/mL), 10 µL sample were added to 2.4 mL buffer, and the mixture was incubated at 37 °C for 15 min. After 15 min incubation, 50 µL of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 20 µL of 75 mM acetylthiocholine iodide (ATCI) were added, and the final mixture was incubated at room temperature for 30 min. Blank was prepared using 10 µL of DMSO instead of the test sample, with all other procedures similar to those used in the case of the sample mixture. Absorbances were measured at 412 nm and 37 °C using polystyrene cuvettes with spectrophotometer (UV-1700, Shimadzu). Experiment was done in triplicate. Galantamine was used as a positive control. Data are expressed as mean ± standard deviation (SD). The inhibition (percent) of AChE was calculated using the following equation:

$$I\ (\%) = 100 - (OD_{sample} / OD_{control}) \times 100$$

4.3. *In silico* Prediction of ADME Parameters

The physicochemical parameters (log P, TPSA, nrotb, molecular weight, number of hydrogen bond donors and acceptors, molecular volume) of the compounds were calculated using Molinspiration software [23-26].

Acknowledgements: This study was supported by Anadolu University Scientific Research Projects Commission under the grant no: 1705S175.

Author contributions: Concept – M.D.A., G.A.Ç., H.E.T.; Design – M.D.A., G.A.Ç., H.E.T.; Supervision – M.D.A.; Resource – M.D.A., G.A.Ç., H.E.T.; Materials – M.D.A., G.A.Ç., H.E.T.; Data Collection and/or Processing - M.D.A., G.A.Ç., H.E.T.; Analysis and/or Interpretation - M.D.A.; Literature Search – M.D.A.; Writing – M.D.A.; Critical Reviews – M.D.A., G.A.Ç., H.E.T.

Conflict of interest statement: The authors declared no conflict of interest.

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