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Design, synthesis and antitumor activities of some novel benzoxazole carbohydrazide derivatives.

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ABSTRACT

Synthesis of novel benzoxazole carbohydrazide derivatives. Compound (**2)** synthesized according known method and it is used as intermediate compound for the synthesis of different new series of acyclic and heterocyclic compounds such as compound **(3)** which obtained at the refluxed of compound (**2)** with carbon disulfide in alcoholic KOH. Also, at the condensation compound **(3)** with hydrazine hydrate, compound (**4)** was proceed. While the addition of carbon disulfide in alcoholic KOH at room temperature to compound **(2)**, compound **(5)** was offered**.** Compound **(5)** acidified with sulfuric acid to yield compound (**6)** which proceed compounds **(7-9)** at using different reagents**.** Compounds **(10a, b)**, were obtained at the reaction of furan-2, 5-dione or/ dihydrofuran-2, 5-dione with compound **(2).** Also, at the condensation of compound **(2)** with chloroacetyl chloride, 2-chloroacetyl compound **(11)** was afforded, which was reacted with thio semicarbazide to give compound **(12)** was obtained. While the Condensation of compound **(2)** with 2-acetylthiophene and 4-flourobenzaldehyde gave compound (**13, 14)** respectively. Refluxing of compound **(2)** with phenyl isocyanate and /or benzyl isothiocyanate, respectively to proceed compounds **(15, 17)**, respectively. At the reaction of both compounds **(15, 17)** with ethyl bromoacetate, compounds **(16, 18)** were obtained. The structures of the newly compounds were confirmed based on different spectroscopic and elemental analyses (IR, ^{1}H, ^{13}C NMR and MS). Selected new compounds were evaluated their activity against a panel of two antitumor cell lines; human pancreatic and epithelial-like cell line (panc-1) and heterogeneous human epithelial colorectal adenocarcinoma (Caco-2) in comparison with the activity of the known Doxorubicin anticancer reference drug. Some of the selected compounds showed promising activity.

Keywords: benzoxazole carbohydrazide**,** 2-acetylthiophene, phenyl isocyanate, benzyl isothiocyanates, Anticancer, Caco-2, panc-1.

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INTRODUCTION

Cancer is a leader disease in cause of death in the world specially developed countries. Surgery is potentially curative, with high risk and its strategy is still based on using only chemotherapeutic drugs or with radiotherapy. Therefore, it is urgent to synthesize and develop new efficient chemotherapeutic drug for cancer treatment. So, it is know that Synthetic organic chemistry [1] is playing a significant role in the development of new drugs. Heterocyclic compounds [2] are the most important in the discovery and development of new drugs, large numbers of heterocyclic compounds having important applications and important intermediates in organic synthesis. Heterocyclic compounds [3] have useful pharmacological activities. Recent observations show that substituted benzoxazole and related heterocycles [4-6], possess potential activity with lower toxicities. Herein we report the synthesis and in vitro growth inhibition characterization of new benzo[d]oxazole derivatives. The in vitro antiproliferative activity of each compound in the study has been determined [7-10] in human pancreatic epithelial-like cell line (panc-1) and heterogeneous human epithelial colorectal adenocarcinoma (Caco-2). The cytotoxic potency of the selected products was studied in comparison to the known anticancer Doxorubicin (DOX) drug.

EXPERIMENTAL

All melting points are uncorrected and were taken on open capillary tubes using electro thermal apparatus 9100. Elemental micro analyses were carried out at micro analytical unit , Central Services Laboratory, National Research Centre, Dokki, Giza -Egypt, using Vario Elementar and were found within + or - 0.5% of the theoretical values. Infrared spectra were recorded on a Jasco FT/IR-6100, Fourier Transform Infrared Spectrometer at cm^{-1} scale using KBr disc technique at the Central Services Lab. NRC, Dokki, Giza, Egypt. ^{1}HNMR spectra were determined by using a JEOL EX-270 NMR Spectrometer at Central Services Lab, NRC. Mass spectra were measured with Finnegan M A T SSQ-7000 mass spectrometer at the Central Services, NRC Dokki, Giza, Egypt. Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel-recoated aluminum sheets (Type 60 F254-Merck, Darmstadt, Germany) and the spots were detected by exposure to UV Lamp at 254 nanometer for few seconds. Nomenclature given for the new compounds are according to the IUPAC System.

General procedures for preparation of ethyl benzo[d]oxazole-2-carboxylate (1)

A mixture of *o*-aminophenol (0.01mol) and diethyl oxalate (0.05mol) was heated under reflux for 48h at 70°C. The reaction mixture monitoring by TLC. The solid product that precipitated after concentration was filtered off, washed with petroleum ether, dried and recrystallized from ethanol. yield 65% as rose crystals, m.p.134- 136 °C, IR (KBr, cm^{-1}): 3045 (CH aromatic), 1624 (C=N), 1666 (C=O); ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 1.38 (t, 3H, CH_3), 4.34 (q, 2H, CH_2), 7.32-7.37 (*m*, 4H, Ar-H); MS (*m/z*, (relative abundance, %)): 191 (M^+, 61); Anal. Calcd. ,for $C_{10}H_9NO_3$: C, 62.82; H, 4.74; N, 7.33; Found: C, 62.80; H, 4.71; N, 7.31.

General procedures for preparation of benzo[d]oxazole-2-carbohydrazide (2)

A mixture of compound **1** (5 mmol) and hydrazine hydrate (5 mmol) in 20mL ethanol was heated under reflux for 15h. The reaction mixture monitoring by TLC. The solid product that precipitated after cooling, was filtered off, washed with alcohol, dried and recrystallized from ethanol; yield 30% as colorless crystals, m.p. 200- 202°C, IR (KBr, cm^{-1}): 3461, 3350, 3190 (NH, NH_2), 1690 (C=O), 1615 (C=N); ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 2.42 (s, 2H, NH_2, exchangeable with D_2O), 7.18 (br. s, 1H, NH, exchangeable with D_2O), 7.28-7.39 (*m*, 4H, Ar-H); MS (*m/z*, (relative abundance, %)): 177 (M^+, 37); Anal. Calcd., for $C_8H_7N_3O_2$: C, 54.24; H, 3.98; N, 23.72; Found: C, 54.22; H, 3.94; N, 23.71.

General procedures for preparation of 5-(benzo[d]oxazol-2-yl)-1, 3, 4-oxadiazole-2(3H)-thione (3)

Carbon disulfide (0.015 mol) was added drop-wise with constant stirring to a solution of compound **2** (0.01mol) in ethanoic potassium hydroxide solution (0.01mole in 5mL). The reaction mixture was heated on water bath at 70°C for 10h until the evolution of H_2S caused. The reaction mixture was concentrated to one fourth of its volume, poured onto ice and acidified dilute HCl. The solid product that precipitated was filtered off, washed with water, dried and recrystallized from ethanol; yield 56% as yellow crystals, m.p.280- 283 °C, IR (KBr, cm^{-1}): 3190 (NH), 1645, 1636 (C=N), 1219 (C=S). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 7.35-7.44 (*m*, 4H,

Ar-H), 9.70 (br. s, 1H, NH, exchangeable with D_2O). MS (*m/z*, (relative abundance, %)): 219 (M^+, 62); Anal. Calcd., for $C_9H_5N_3SO_2$: C, 49.31; H, 2.30; N, 19.17; Found: C, 49.30; H, 2.26; N, 19.15.

General procedures for preparation of 4-amino-3-(benzo[d]oxazol-2-yl)-1H-1, 2, 4-triazole-5(4H)-thione (4)

To a solution of compound **3** (0.01 mol) in (5mL) DMF, hydrazine hydrate (0.03mol) in (10 ml) absolute ethanol was added drop-wise with stirring. The reaction mixture was reflux for 9h. The reaction mixture monitoring by TLC. The precipitate was formed, was filtered off, washed with ethanol, dried and recrystallized from acetone; yield 32% as pale yellow crystals, m.p. 158- 160 ^oC, IR (KBr, cm^{-1}): 3440-3330, 3290 (NH, NH_2), 1648, 1636 (C=N), 1224 (C=S). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 3.11 (s, 2H, NH_2, exchangeable with D_2O), 7.36-7.42 (*m*, 4H, Ar-H), 8.72 (br. s, 1H, NH, exchangeable with D_2O); ^{13}C NMR (125 MHz, DMSO-d_6, δ / ppm): 172.9 (C=S), 161.0, 135.0 (C=N), 152.0, 138.2, 125.4, 124.3, 118.2, 111.23 (CH, aromatic). MS (*m/z*, (relative abundance, %)): 231 (M^+-2, 30); Anal. Calcd, for $C_9H_7N_5OS$: C, 46.34; H, 3.02; N, 30.03; Found: C, 46.30; H, 3.04; N, 30.00.

General procedures for preparation of potassium 2-(benzo[d]oxazole-2-carbonyl) hydrazine carbodithioate (5) and 5-(benzo[d]oxazol-2-yl)-1, 3, 4-thiadiazole-2-thiol (6)

Carbon disulfide (0.005 mol) was added drop-wise to a mixture of KOH (0.0025 mol) in absolute ethanol (50mL) and compound **2** (0.0025 mol). The reaction mixture stirred at r.t. for 28h. The solid product that precipitated was filtered off, washed with petroleum ether and ethanol, dried and recrystallized from ethanol to give compound **5**.

Compound **5** (0.01 mol) was added portion-wise to very cold conc. sulfuric acid (20mL) with stirring at r.t. for 5h. The reaction mixture was poured onto ice water and neutralized with Ammonium hydroxide. The solid product was filtered off, washed with water, dried and recrystallized from ethanol to give compound **6**; yield 46% as buff crystals, m.p233-235^oC, IR (KBr, cm^{-1}): 2500 (SH), 1660, 1648, 1624 (C=N). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 1.95 (br. s, 1H, SH, exchangeable with D_2O), 7.32-7.41 (*m*, 4H, Ar-H); ^{13}C NMR (125 MHz, DMSO-d_6, δ / ppm): 191.0 (N=C-S), 151.9, 151.8 (C=N), 152.0, 138.3, 125.5, 124.4, 118.3, 111.3 (CH, aromatic). MS (*m/z*, (relative abundance, %)): 237 (M^++2, 31); Anal. Calcd., for $C_9H_5N_3S_2O$: C, 45.94; H, 2.14; N, 17.86; Found: C, 45.96; H, 2.15; N, 17.84.

General procedures for preparation of 2-(5-(methylthio)-1, 3, 4-thiadiazol-2-yl) benzo[d]oxazole (7)

To a mixture of KOH (0.006 mol) in absolute ethanol (30mL) and compound **6** (0.002 mol), add methyl iodide (2g) drop-wise with stirring. The reaction mixture was heated at 60^oC on water bath for 28h. The reaction mixture was poured onto cold water. The precipitated product was filtered off, dried and recrystallized from ethanol to give compound **7**; yield 50% as buff crystals, m.p211-213^oC, IR (KBr, cm^{-1}): 1660, 1642, 1623 (C=N). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 2.52 (*s*, 3H, CH_3), 7.36-7.44 (*m*, 4H, Ar-H). MS (*m/z*, (relative abundance, %)): 249 (M^+, 27); Anal. Calcd., for $C_{10}H_7N_3S_2O$: C, 48.18; H, 2.83; N, 16.85; Found: C, 48.17; H, 2.79; N, 16.84.

General procedures for preparation of 5-(benzo[d]oxazol-2-yl)-3-methyl-1, 3, 4-thiadiazole-2(3H)-thione (8)

The foregoing method was applied using stirring at r.t. for 8h and recrystallized from ethyl acetate/petroleum ether to give compound **8**; yield 42% as pale brown crystals, m.p187-189^oC, IR (KBr, cm^{-1}): 1660, 1623 (C=N), 1224 (C=S). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 3.43 (*s*, 3H, CH_3), 7.35-7.44 (*m*, 4H, Ar-H). ^{13}C NMR (125 MHz, DMSO-d_6, δ / ppm): 197.6 (C=S), 151.5, 145.9 (C=N), 152.0, 138.3, 125.5, 124.4, 118.2, 111.2 (CH, aromatic), 39.8 (CH_3). MS (*m/z*, (relative abundance, %)): 249 (M^+, 27); Anal. Calcd., for $C_{10}H_7N_3S_2O$: C, 48.18; H, 2.83; N, 16.85; Found: C, 48.14; H, 2.77; N, 16.84.

General procedures for preparation of compound 9a, b

To a saturated solution of sodium metal in absolute ethanol (20mL), compound **6** (0.01mol) was added and the appropriate bromoacetyl namely, 1, 2, 3, 4-tetrahydronaphthalene and/or bromoacetyl naphthalene with stirring at r.t. for 35h. The reaction mixture was poured onto cold water. The precipitated product was filtered off, dried and recrystallized from proper solvent to give compounds **9a,b.**

2-((5-(Benzo[d]oxazol-2-yl)-1, 3, 4-thiadiazol-2-yl) thio)-1-(1, 2, 3, 4-tetrahydronaphthalen-1-yl) ethanone (9a)

Yield 45% as brown crystals (acetone), m.p219-221°C, IR (KBr, cm^{-1}): 1700 (C=O), 1656, 1640 (C=N). ^{1}H-NMR (270MHz, DMSO-d_6, δ / ppm): 1.70, 2.12, 2.70 (m, 6H, cyclic CH_2), 3.70 (*m*, 1H, cyclic CH), 4.15 (*s*, 2H, CH_2), 7.00-7.44 (*m*, 8H, Ar-H). MS (*m/z*, (relative abundance, %)): 407 (M^+, 20); Anal. Calcd. , for $C_{21}H_{17}N_3O_2S_2$: C, 61.89; H, 4.20; N, 10.31; Found: C, 61.90; H, 4.22; N, 10.30.

2-((5-(benzo[d]oxazol-2-yl)-1, 3, 4-thiadiazol-2-yl) thio)-1-(naphthalen-1-yl) ethanone (9b)

Yield 50% as dark brown crystals (ethanol), m.p>300°C, IR (KBr, cm^{-1}): 1696 (C=O), 1660, 1640, 1620 (C=N). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 4.30 (*s*, 2H, CH_2), 7.33-8.34 (*m*, 11H, Ar-H). MS (*m/z*, (relative abundance, %)): 403 (M^+, 20); Anal. Calcd., for $C_{21}H_{13}N_3O_2S_2$: C, 62.51; H, 3.25; N, 10.41; Found: C, 62.50; H, 3.23; N, 10.45.

General procedures for preparation of compound 10a, b

A mixture of compound **2** (0.01 mol), and the appropriate anhydride namely; succinic or/ maleic (0.01mol) in glacial acetic acid was refluxed for 12h. The reaction mixture monitoring by TLC. The glacial acetic acid was removed under vacuum and the residue was poured onto cold water. The formed precipitated was filtered off, washed several times with water, dried and recrystallized from proper solvent to give compounds **10a,b.**

N-(2, 5-dioxopyrrolidin-1-yl) benzo[d]oxazole-2-carboxamide (10a)

Yield 40% as dark brown crystals (ethanol), m.p148-50°C, IR (KBr, cm^{-1}): 3204 (NH), 1705, 1700, 1682 (C=O), 1640 (C=N). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 2.94 (s, 4H, CH_2- CH_2), 7.12 (br. s, 1H, NH, exchangeable with D_2O), 7.30-7.39 (*m*, 4H, Ar-H); ^{13}C NMR (125 MHz, DMSO-d_6, δ / ppm): 174.1, 151.3 (C=O), 146.6 (C=N), 152.0, 138.3, 125.5, 124.4, 118.2, 111.2 (CH, aromatic), 26.6 (CH_2). MS (*m/z*, (relative abundance, %)): 260 (M^++1, 61); Anal. Calcd., for $C_{12}H_9N_3O_4$: C, 55.60; H, 3.50; N, 16.21; Found: C, 55.57; H, 3.46; N, 16.20.

N-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) benzo[d]oxazole-2-carboxamide (10b)

Yield 43% as brown crystals (ethanol), m.p170-172°C, IR (KBr, cm^{-1}): 3214 (NH), 1710, 1700, 1685 (C=O), 1640 (C=N). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 6.93 (s, 1H, NH, exchangeable with D_2O), 7.34-7.45 (*m*, 4H, Ar-H), 7.52 (s, 2H, CH=CH); ^{13}C NMR (125 MHz, DMSO-d_6, δ / ppm): 170.6, 151.3 (C=O), 146.6 (C=N), 137.5 (CH), 152.0, 138.3, 125.5, 124.4, 118.2, 111.2 (CH, aromatic). MS (*m/z*, (relative abundance, %)): 255 (M^+-2, 31); Anal. Calcd., for $C_{12}H_7N_3O_4$: C, 56.04; H, 2.74; N, 16.34; Found: C, 56.00; H, 2.72; N, 16.32.

General procedures for preparation of N'-(2-chloroacetyl) benzo[d]oxazole-2-carbohydrazide (11)

To a solution of compound **2** (0.01 mol) and anhydrous K_2CO_3 (0.01 mol) in 15 mL acetone, chloroacetyl chloride (0.01 mol) was added drop-wise with stirring. The reaction mixture was stirred for 10h at r.t. The reaction mixture monitoring by TLC. The excess anhydrous K_2CO_3 was filtered and the filtrate concentrated under vaccum the precipitate washed with petroleum ether .Yellow precipitated was obtained and recrystallized from methanol; yield 40% as yellow crystals, m.p. 144-146°C, IR (KBr, cm^{-1}): 3240, 3230 (br. NHs), 1696, 1684 (C=O), 1630 (C=N). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 3.55 (s, 2H, CH_2), 7.30-7.38 (*m*, 4H, Ar-H), 8.30 (br., 1H, NH, exchangeable with D_2O), 8.70 (br. s, 1H, NH, exchangeable with D_2O). MS (*m/z*, (relative abundance, %)): 253 (M^+, 27); Anal. Calcd., for $C_{10}H_8ClN_3O_3$: C, 47.35; H, 3.18; N, 16.57; Found: C, 47.33; H, 3.20; N, 16.55.

General procedures for preparation of N'-(3-thioxo-1, 2, 3, 6-tetrahydro-1, 2, 4-triazin-5-yl) benzo[d]oxazole-2-carbohydrazide (12)

To a solution of compound **11** (0.01 mol) and anhydrous K_2CO_3 (0.01 mol) in 15 mL DMF, thio semicarbazide (0.01 mol) was added. The reaction mixture was refluxed for 15h. The reaction mixture left to

cool, then poured into ice water, monitoring by TLC. The precipitated product was collected by filtration and recrystallized from ethanol; yield 37% as brown crystals, m.p.177-179 °C, IR (KBr, cm^{-1}): 3340, 3320, 3310, 3275 (br. NHs), 1685 (C=O), 1635, 1629 (C=N), 1231 (C=S). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 3.48 (s, 2H, CH_2), 6.90 (br,s 1H, NH, exchangeable with D_2O), 7.31-7.39 (*m*, 4H, Ar-H), 8.00 (s, 1H, NH, exchangeable with D_2O), 8.45 (br. s, 1H, NH, exchangeable with D_2O). MS (*m/z*, (relative abundance, %)): 288 (M^+-2, 44); Anal. Calcd., for $C_{11}H_{10}N_6O_2S$: C, 45.51; H, 3.47; N, 28.95; Found: C, 45.48; H, 3.42; N, 28.92.

General procedures for preparation of N'-(1-(thiophen-2-yl) ethylene) benzo[d]oxazole-2-carbohydrazide (13)

A mixture of compound **2** (0.01 mol), and 2-acetyl thiophene (0.01mol) in absolute ethanol (20mL) and few drops of TEA was refluxed for 9h. The reaction mixture monitoring by TLC, was allowed to cool. The formed precipitated was filtered off, dried and recrystallized from acetone to give compound **13**; yield 45% as brownish yellow crystals, m.p. >300°C, IR (KBr, cm^{-1}): 3254 (br. NH), 1675 (C=O), 1642, 1635 (C=N. ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 2.43 (s, 3H, CH_3), 7.12-7.14 (t, 1H, thiophene H), 7.34-7.42 (*m*, 4H, Ar-H), 7.34, 7.48 (dd, 2H, thiophene H), 9.45 (br. s, 1H, NH, exchangeable with D_2O). ^{13}C NMR (125 MHz, DMSO-d_6, δ / ppm): 153.5 (C=O), 148.3, 142.2 (C=N), 152.0, 138.3, 130.1, 127.7, 127.4, 125.5, 124.4, 118.3, 111.2 (CH, aromatic), 14.8 (CH_3). MS (*m/z*, (relative abundance, %)): 283 (M^+-2, 44); Anal. Calcd. ,for $C_{14}H_{11}N_3O_2S$: C, 58.93; H, 3.89; N, 14.73; Found: C, 58.94; H, 3.87; N, 14.71.

General procedures for preparation of N'-(4-fluorobenzylidene) benzo[d]oxazole-2-carbohydrazide (14)

A mixture of compound **2** (0.01 mol), and p-fluorobenzaldehyde (0.01mol) in absolute ethanol (20mL) in presence of few drops of triethyl amine was refluxed for 10h. The reaction mixture monitoring by TLC and was allowed to cool. The formed precipitated was filtered off, dried and recrystallized from ethanol to give compound **14**; yield 41% as brown crystals, m.p.190-192 °C, IR (KBr, cm^{-1}): 3190 (br. NH), 1680 (C=O), 1637, 1625 (C=N), 1600 (C=C). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 7.16-7.37 (*m*, 8H, Ar-H), 7.71 (s, 1H, CH=N), 9.50 (br. s, 1H, NH, exchangeable with D_2O). ^{13}C NMR (125 MHz, DMSO-d_6, δ / ppm): 153.5 (C=O), 149.3, 148.3 (C=N), 161.3, 152.0, 138.3, 131.5, 130.4, 130.1, 127.7, 127.4, 125.5, 124.4, 118.3, 115.5, 111.2 (CH, aromatic). MS (*m/z*, (relative abundance, %)): 285 (M^++2, 49); Anal. Calcd., for $C_{15}H_{10}FN_3O_2$: C, 63.60; H, 3.56; N, 14.83; Found: C, 63.58; H, 3.52; N, 14.85.

General procedures for preparation of compounds 15, 17

A mixture of compound **2** (0.01 mol), and the appropriate cyanate namely; phenyl isocyanate and/or benzyl isothiocyanate (0.01 mol) in absolute ethanol (20mL) in presence of few drops of acetic acid was refluxed for 8h. The reaction mixture monitoring by TLC. The formed precipitated was filtered off, dried and recrystallized from the proper solvent to give compounds **15** or **17**.

2-(Benzo[d]oxazole-2-carbonyl)-N-phenyl hydrazine carboxamide (15)

Yield 63% as brown crystals (ethanol), m.p.224-226 °C, IR (KBr, cm^{-1}): 3351, 3300, 3285 (br. NHs), 1690, 1674 (C=O), 1640 (C=N). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 6.65 (br. s, 1H, NH, exchangeable with D_2O), 6.94-7.43 (*m*, 9H, Ar-H), 8.30 (br. s, 1H, NH, exchangeable with D_2O), 9.75 (br. s, 1H, NH, exchangeable with D_2O). MS (*m/z*, (relative abundance, %)): 296 (M^+, 24); Anal. Calcd., for $C_{15}H_{12}N_4O_3$: C, 60.81; H, 4.08; N, 18.91; Found: C, 60.79; H, 4.10; N, 18.89.

2-(Benzo[d]oxazole-2-carbonyl)-N-benzyl hydrazine carbothioamide (17)

Yield 63% as dark brown crystals (benzene), m.p. 211-213°C, IR (KBr, cm^{-1}): 3300, 3219, 3186 (br. NHs), 1687 (C=O), 1634 (C=N), 1219 (C=S). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 4.75 (s, 2H, CH_2), 7.00 (br. s, 1H, NH, exchangeable with D_2O), 7.20-7.40 (*m*, 9H, Ar-H), 9.30 (br. s, 1H, NH, exchangeable with D_2O), 9.85 (br. s, 1H, NH, exchangeable with D_2O). MS (*m/z*, (relative abundance, %)): 326 (M^+, 71); Anal. Calcd., for $C_{16}H_{14}N_4O_2S$: C, 58.88; H, 4.32; N, 17.17; Found: C, 58.90; H, 4.30; N, 17.20.

General procedures for preparation of compounds (16, 18)

A mixture of compound **15** and/or **17** (0.01 mol) and ethyl bromoacetate (0.01 mol) in absolute ethanol (20mL) in presence of anhydrous sodium acetate (0.02mol) was refluxed for 8h. The reaction mixture monitoring by TLC and the reaction mixture diluted with water and leave overnight. The formed precipitated was filtered off, dried and recrystallized from the proper solvent to give compounds **16** or **18**.

Ethyl 2-(2-(benzo[d]oxazole-2-carbonyl)-1-(phenyl carbamoyl) hydrazinyl) acetate (16)

Yield 43% as dark brown crystals (acetone), m.p. >300°C, IR (KBr, cm^{-1}): 3220, 3195 (br. NHs), 1700, 1670, 1655 (C=O), 1630 (C=N). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 1.38 (t, 3H, CH_3), 4.20 (q, 2H, CH_2), 4.36 (s, 2H, CH_2), 6.95-7.50 (*m*, 9H, Ar-H), 8.10 (br. s, 1H, NH, exchangeable with D_2O), 9.35 (br. s, 1H, NH, exchangeable with D_2O). MS (*m/z*, (relative abundance, %)): 382 (M^+, 21); Anal. Calcd., ,for $C_{19}H_{18}N_4O_5$: C, 59.68; H, 4.74; N, 14.65; Found: C, 59.70; H, 4.72; N, 14.63.

N'-(3-benzyl-4-oxothiazolidin-2-ylidene) benzo[d]oxazole-2-carbohydrazide (18)

Yield 40% as brown crystals (acetone), m.p.>300 °C, IR (KBr, cm^{-1}): 3285 (br. NH), 1695, 1677 (C=O), 1640, 1624 (C=N). ^{1}H-NMR (270 MHz, DMSO-d_6, δ / ppm): 4.02 (s, 2H, CH_2), 4.70 (s, 2H, CH_2), 7.23-7.42 (*m*, 9H, Ar-H), 9.35 (br. s, 1H, NH, exchangeable with D_2O). MS (*m/z*, (relative abundance, %)): 366 (M^+, 21); Anal. Calcd., for $C_{18}H_{14}N_4O_3S$: C, 59.01; H, 3.85; N, 15.29; Found: C, 59.00; H, 3.83; N, 15.31.

Anticancer evaluation

Cell culture and treatment

All reagents were handled as previously described in our work [11–16] in a sterile fume hood. DMEM medium and fetal bovine serum (FBS) were purchased from Gibco; phosphate-buffered saline pH 7.4 (PBS) and trypsin-EDTA were obtained from Sigma–Aldrich. Alamar Blue or Resazurin (Promega, Mannheim, Germany) reduction assay was used to assess the cytotoxicity of the studied samples. The growth medium (DMEM medium with 10 % FBS, 100 U/mL penicillin, and 100 mg/L streptomycin), and Alamar Blue were stored at 48°C, while trypsin–EDTA and FBS were stored frozen at -208 C and thawed before use; PBS was stored at room temperature. Caco-2 and Panc-1 were obtained from the German Cancer Research Center (DKFZ). Cells were cultured in 50 cm^2 culture flasks (Corning) using DMEM medium supplemented with 10 % FBS, penicillin (100 IU/ mL), and streptomycin (100 mg/mL). The culture was maintained at 37°C in an atmosphere of 5 % CO_2 and 95 % relative humidity. The cells were transferred to a new flask every 2 days and treated with trypsin-EDTA to detach them from the flask. Cells were counted under a microscope using a hem cytometer (Hausser Scientific). Cell solutions were diluted with growth medium to a concentration of ($1*10^5$ cells/mL) and transferred to a 96-well plate, and treated with gradient concentrations of test compounds.

Resazurin cell growth inhibit ion assay

Alamar Blue or Resazurin (Promega, Mannheim, Germany) reduction assay was used to assess the cytotoxicity of the studied samples. The assay tests the cellular viability and mitochondrial function. Briefly, adherent cells were grown in tissue culture flasks, and then harvested by treating the flasks with 0.025 % trypsin and 0.25 mM EDTA for 5 min. Once detached, cells were washed, counted, and an aliquot (5 9 103 cells) was placed in each well of a 96-well cell culture plate in a total volume of 100 lL. Cells were allowed to attach overnight and then treated with samples. The final concentration of samples ranged from 0–100 lM. After 48 h, 20 lL Resazurin 0.01 % w/v solution was added to each well and the plates were incubated at 37 C for 1–2 h. Fluorescence was measured on an automated 96-well Infinite M2000 ProTM plate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. After 48 h incubation, plates were treated with resazurin solution as above mentioned. Doxorubicin was used as a positive control. Each assay was done at least three times, with two replicates each. The viability was compared based on a comparison with untreated cells. IC_{50} (on cancer cells) were the concentration of the sample required to inhibit 50 % of the cell proliferation and were calculated from a calibration curve by a linear regression using Microsoft Excel.

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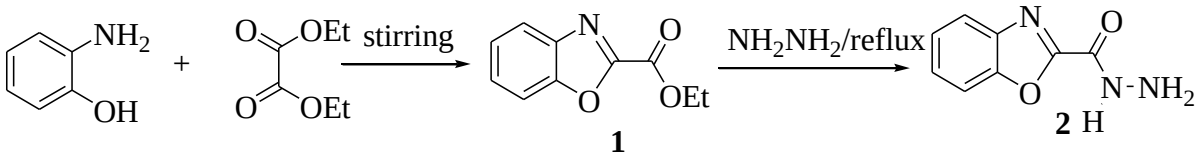
RESULTS AND DISCUSSIONS

Chemistry

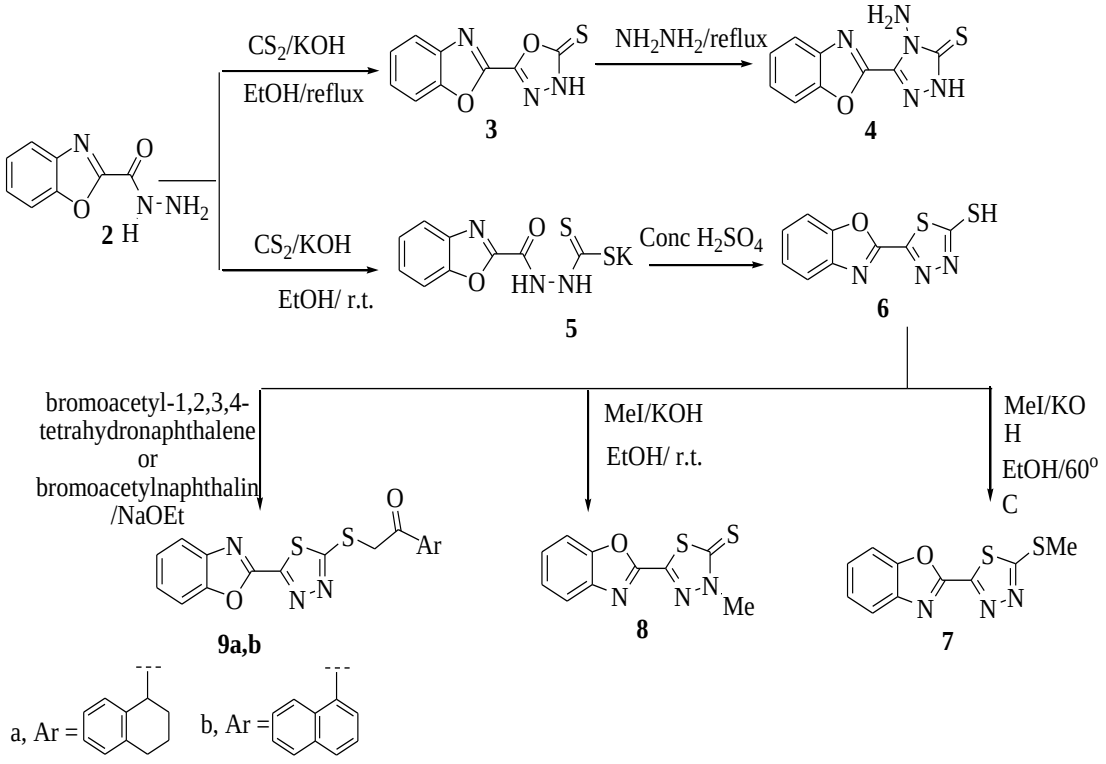
Hydrazones [17] have wide interest because of their diverse biological applications. In addition, hydrazide-Hydrazones were reported to elicit anticancer and antiHIV properties and hence they have gained an important place in medicinal chemistry [18]. Benzo[d]oxazole-2-carbohydrazide (**2)** was prepared by refluxing of ethyl benzo[d]oxazole-2-carboxylate (**1**) with hydrazine hydrate and it is used as key intermediate compound for the synthesis of different new series of acyclic and heterocyclic compounds (Scheme 1).



Scheme 1

Triazole compounds [19] used as medicinal drugs. So, When compound (**2)** reacted with carbon disulfide in alcoholic KOH under reflux temperature [20,21], it gave 1, 3, 4-oxadiazole-2(3H)-thione derivative (**3)** (Scheme 2). This compound reacted with hydrazine hydrate at reflux temperature to give 1, 2, 4-triazole-5(4H)-thione derivative (**4)** in good yield (Scheme 2). IR spectrum showed (C=S) at 1219 in compound (**3)** and at 1224 in compound (**4)**. In addition, absorption band of NH group appeared at 3190 (NH) in compound (**3)**, while NH, NH_2 groups appeared at 3440, 3330, 3290 in compound (**4)**.

In the same manner, compound (**2)** reacted with carbon disulfide in alcoholic KOH at room temperature, potassium 2-(benzo[d]oxazole-2-carbonyl) hydrazine carbodithioate (**5**) was obtained as buff crystals in a good yield. Compound **5** acidified with sulfuric acid to give 5-(benzo[d]oxazol-2-yl)-1, 3, 4-thiadiazole-2-thiol (**6**) (Scheme 2). ^{1}H-NMR of compound **6** had a singlet signal at δ 1.95 of (SH) which is exchangeable with D_2O.



Scheme 2

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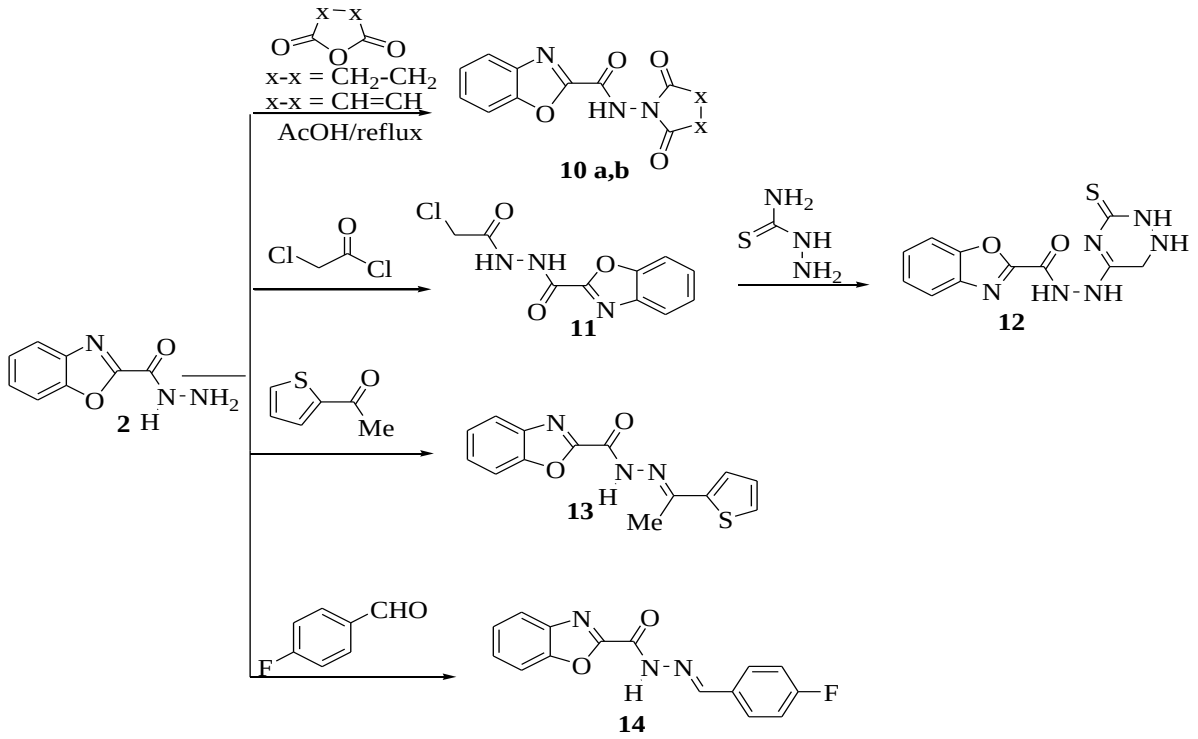
Compound **(6)** was reacted with methyl iodide in alcoholic KOH under reflux temperature to give methylated derivative (**7**), while it was stirred at room temperature, 5-(benzo[d]oxazol-2-yl)-3-methyl-1,3,4-thiadiazole-2(3H)-thione **(8)** had been obtained (Scheme 2). IR spectrum of compounds (**7)** and (**8)** showed three absorption bands at 1660, 1642, 1623 of (C=N) and at two absorption bands at 1660, 1623 (C=N), 1224 (C=S) respectively.

Also, when compound (**6)** was allowed to react with bromoacetyl-1, 2, 3, 4-tetrahydronaphthalene and/or bromoacetyl naphthalene in presence of sodium ethoxide solution at reflux temperature, it had given compounds (**9a, b)** respectively (Scheme 2). ^{1}H-NMR of compound (**9a)** showed multiple signals at δ 1.70, 2.12, 2.70 ppm of (cyclic CH_2), 3.70 ppm of (cyclic CH), and 7.00-7.44 ppm of aromatic protons.

Benzo[d]oxazole-2-carbohydrazide **2** reacted with succinic anhydride or/ maleic anhydride in acetic acid solution at reflux temperature to give 2,5-dioxopyrrolidin and/or 2, 5-dioxo-2, 5-dihydro-1H-pyrrole derivatives (**10a, b)** respectively (Scheme 3). ^{1}H-NMR of compound (**10a**) showed singlet signals at δ 2.94 (cyclic CH_2-CH_2), while at compound (**10b)**; it appeared as a two doublet at δ 7.30 and at 7.38 ppm (2 cyclic CH). Also, reaction of compound (**2)** with chloroacetyl chloride [22] had been investigated in dry acetone in presence of anhydrous potassium carbonate to obtain *N'*-(2-chloroacetyl) benzo[d]oxazole-2-carbohydrazide (**11**) which was reacted with thio semicarbazide in DMF solution at reflux temperature for 15 h to give N'-(3-thioxo-1,2,3,6-tetrahydro-1,2,4-triazin-5-yl)benzo[d]oxazole-2-carbohydrazide (**12**) (Scheme 3). IR spectrum of compound (**11)** was showed broad bands at 3240, 3230 (br. NHs), 1696, 1684 (C=O), and 1630 (C=N), while IR spectrum of compound (**12)** showed Absorption bands at 3397, 3390, 3310, 3275 (br. NHs), 1685 (C=O), 1635, 1629 (C=N) and 1231 (C=S).



Scheme 3

Condensation of benzo[d]oxazole-2-carbohydrazide (**2)** with 2-acetylthiophene and 4-flourobenzaldehyde [23] in absolute ethanol solution at reflux temperature in the presence of few drops of pyridine or triethyl amine was investigated to yield the compounds (**13)** and (**14)** respectively (Scheme 3).

^{1}H-NMR spectrum of compound (**13)** showed singlet signal at δ 2.43 of methyl group, doublet signals at δ 7.34, 7.48 (thiophene H), multiplet signal at 7.12, 7.14 (thiophene H), singlet signal at 2.43(CH_3), 7.34-7.52 and singlet broad signal at δ 9.45 for (NH) which is exchangeable with D_2O. In addition, ^{1}H-NMR spectrum of compound (**14)** showed singlet signal δ 7.71 for (CH=N) and singlet br. signal at 9.45 (NH).

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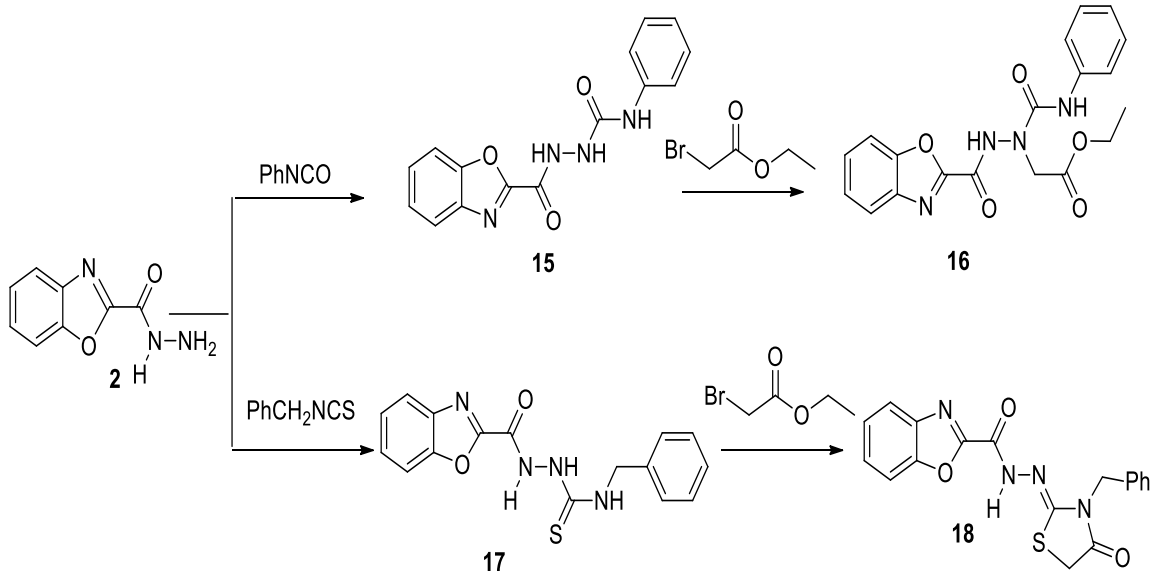
N-phenyl hydrazine carboxamide derivative (**15)** and/or *N*-benzyl hydrazine carbothioamide derivative (**17)** were formed through reaction of benzo[d]oxazole-2-carbohydrazide (**2)** with phenyl isocyanate and /or benzyl isothiocyanate, respectively in the presence of acetic acid in absolute ethanol solution (Scheme 4). Mass spectrum of compound (**15)** showed ion peak at 296 (M^+, 24%) while in compound (**17)**, it showed mass ion peak at 329 (M^+, 71%).



Scheme 4

Compound (**15)** and /or (**17)** were reacted with ethyl bromoacetate in in the presence of anhydrous sodium acetate in absolute ethanol solution to give hydrazinyl acetate derivative (**16**) and/or 4-oxothiazolidine derivative (**18)** (Scheme 4).

^{1}H-NMR spectrum of compound (**16)** showed triplet signal at δ 1.38 of methyl group and quartet signal at δ 4.20 (CH_2) with singlet signal at δ 4.36 (CH_2). The NH group appeared as two broad singlet signal at δ 8.10 and δ 9.35, which are exchangeable with D_2O. Also, ^{1}H-NMR spectrum of compound (**18)** showed two singlet signals at δ 4.02 (CH_2) and δ 4.70 (CH_2).

The assignments of all newly synthesized compounds were confirmed on the bases of elemental and spectroscopic analyses (IR, ^{1}H, ^{13}C NMR, mass spectra).

ANTICANCER SCREENING

Some of the synthesized compound were screened for their *in-vitro* cytotoxic and growth inhibitory activity against two different types of carcinoma cell lines namely, human pancreatic and epithelial-like cell line (panc-1) and heterogeneous human epithelial colorectal adenocarcinoma (Caco-2) in comparison with the activity of the known anticancer reference drug; Doxorubicin drug. The cytotoxic activity of our tested compounds was expressed as IC_{50} values.

As shown in table 1, In Caco-2 cells, all of tested compounds showed antitumor activity with IC_{50} values ranged from 9.441±3.425- 55.589±µµ. the presence of six membered heterocyclic ring connected to carbohydrazide as compound (**12)** produced more cytotoxic potency than that of Doxorubicin drug. (IC_{50}: 9.441 and 27.40 µµ, respectively). A slight decrease in the activity was observed by the derivatives (**3**, **4**, **6**, **7**, **13** and **18)**, this results may be due to the presence of five-membered ring connected to carbohydrazide. Compounds (**2**, **8**, **11**, **16** and **17)** exhibited remarkable decrease in their cytotoxic activities (IC_{50}: 31.30- 55.58 µµ) less than Doxorubicin drug.

Also, as shown in table 1, panc-1 cells, all of tested compounds showed antitumor activity with IC_{50} values ranged from 19.65±5.12-75.292±4.53µµ. Compound (**18)** showed more cytotoxic potency than

Doxorubicin drug (high efficiency against Caco-2 cells) and also, it showed higher activity than Doxorubicin drug and panc-1 cells (IC_{50}: 19.65 and 24.10 μμ).

Compound (**13)** has higher cytotoxic activity than Doxorubicin drug panc-1 cells carcinoma cell lines (IC_{50}: 24.85±2.33μμ).The rest derivatives showed weak cytotoxic activity. The results that presented of the synthesized compounds are mostly effective as cytotoxic agents against Caco-2 carcinoma cell lines; however, they have low potency against panc-1 carcinoma cell lines.

Table 1: *In vitro* anticancer activity of the tested compounds against Caco-2 and Panc-1 cell lines

Compound no.	Cell lines	
	Caco-2 IC_{50} μμ	panc-1 IC_{50} μμ
Doxorubicin	27.40±0.42	24.10±0.35
2	31.30±3.75	64.25±1.44
3	23.96±6.12	49.38±1.72
4	23.57±3.77	68.29±1.12
6	21.11±14.01	49.14±2.29
7	24.60±2.31	53.12±5.51
8	42.19±8.10	36.11±4.41
11	35.58±4.9	52.46±2.16
12	9.441±3.11	> 100
13	15.40±4.10	24.85±2.33
16	32.36±1.87	68.31±5.12
17	39.53±7.78	75.22±3.55
18	13.10±2.40	19.65±5.53

CONCLUSION

This study represented a good method for synthesis and characterization of benzo[d]oxazole-2-carbohydrazide derivatives. Some of the novel compounds were selected as reprehensive examples to examine their cytotoxic activity against two different types of carcinoma cell lines of human pancreatic and epithelial-like cell line (panc-1) and heterogeneous human epithelial colorectal adenocarcinoma (Caco-2) using Doxorubicin as a reference drug. The obtained results exhibited that that tested compounds are more active against Caco-2 than panc-1 cell lines.

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