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To link to this article: http://dx.doi.org/10.3109/14756366.2013.765416

Published online: 08 Feb 2013.

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Synthesis and cytotoxicity studies of novel benzhydrylpiperazine carboxamide and thioamide derivatives

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Abstract

Synthesis and cytotoxic activities of 32 benzhydrylpiperazine derivatives with carboxamide and thioamide moieties were reported. \textit{In vitro} cytotoxic activities of compounds were screened against hepatocellular (HUH-7), breast (MCF-7) and colorectal (HCT-116) cancer cell lines by sulphorhodamine B assay. In general, 4-chlorobenzhydrylpiperazine derivatives were more cytotoxic than other compounds. In addition, thioamide derivatives (6a–g) have higher growth inhibition than their carboxamide analogs.

Keywords

Benzhydrylpiperazine, cytotoxicity, isocyanate, isothiocyanate, sulphorhodamine B

Introduction

Cancer is the disease resulting from abnormal cells with abilities of uncontrolled dividing and invasion to other tissues through blood and lymph systems. Recently advanced treatment opportunities are unable to overcome the major problems of chemotherapy such as drug resistance and severe side effects due to the lack of specificity. Regarding issues lead the researchers to develop varying drug-like compounds targeting cancer.

Piperazine-1-carboxamides have diverse actions such as antagonism of CB\textsubscript{1}, human CCR2 chemokine, androgen and vanilloid receptors or inhibition of PDGFR phosphorylation\textsuperscript{1–5}.

Benzhydrylpiperazine scaffold is well known for its antihistaminic mimic\textsuperscript{6–11}. Furthermore calcium channel blocking\textsuperscript{12–19}, dopaminergic\textsuperscript{20–23}, antimicrobial\textsuperscript{24–41} and antiviral\textsuperscript{42,43} activities are often mentioned in literature.

Anticancer activity of benzhydrylpiperazines has recently advanced\textsuperscript{44–51}. Kumar et al. have performed cytotoxicity assays to several 1-benzhydrylpiperazine derivatives substituted with variable sulfonyl chlorides, acid chlorides and isothiocyanates. These derivatives have potent cytotoxicity over breast cancer (MCF-7), hepatocellular (HepG-2), cervix (HeLa) and colon carcinoma (HT-29) cell lines\textsuperscript{44}. Yarim et al., also performed cytotoxicity screenings for some 4-chlorobenzhydrylpiperazines substituted with variable benzoyl chloride derivatives and reported their high activities against liver (HUH-7), FOCUS, MAHLAVU, HepG-2, Hep-3B), breast (MCF-7, BT20, T47D, CAMA-1), colon (HCT-116), gastric (KATO-3) and endometrial (MFE-296) cancer cell lines\textsuperscript{45}. In addition, our work group has recently reported a study in which sulfonamide and benzamide derivatives of benzhydrylpiperazines were discussed for their cytotoxicities against HUH-7, MCF-7 and HCT-116 cancer cell lines\textsuperscript{52}.

In this study, we reported the synthesis, purification and characterization of some novel compounds bearing benzhydrylpiperazine backbone. Those compounds were tested for their cytotoxic activities against hepatocellular (HUH-7), breast (MCF-7) and colorectal (HCT-116) cancer cell lines with sulphorhodamine B (SRB) assay. We aimed to develop a structure activity relationship for benzhydrylpiperazine derivatives in accordance with their cytotoxic activity results.

Materials and methods

Chemistry

All chemicals and reagents used in the current study were of analytical grade. The reactions were monitored by thin layer chromatography (TLC) on Merck pre-coated silica GF254 plates (Merck KGaA, Darmstadt, Germany). Melting points (\textdegree{}C) of the compounds were determined by using a Mettler Toledo FP62 capillary melting point apparatus (Mettler-Toledo, Greifensee, Switzerland) and are uncorrected. Ultraviolet spectra were recorded with Agilent 8453 UV-Visible Spectrophotometer (Agilent Technologies, Santa Clara, CA). Infrared spectra were recorded on a Perkin-Elmer Spectrum One Series FT-IR apparatus (Version 5.0.1, Perkin Elmer, Norwalk, CT), using potassium bromide pellets, the frequencies were expressed in cm\textsuperscript{-1}. The \textsuperscript{1}H- and \textsuperscript{13}C-NMR spectra were recorded with a Varian Mercury 400 FT-NMR spectrometer (Varian Inc., Palo Alto, CA), using tetramethylsilane (TMS) as the internal reference, with dimethylsulfoxide (DMSO-d\textsubscript{6}) as solvent, the chemical shifts were reported in parts per million (ppm). Coupling constants were reported in Hertz (Hz) The mass spectra were recorded with a Waters 2695 Alliance Micromass ZQ LC/MS instrument (Waters Corp., Milford, MA). Elemental analyses were performed on...
General procedure for preparation of benzhydrole derivatives

Ten millimoles (2.2 g) of benzophenone was dissolved in 10 ml of ethanol. In a separate flask, 11 mmol (0.4 g) of sodium borohydride (NaBH₄) was dissolved in 2 ml of ethanol. Sodium borohydride solution was slowly added to benzophenone solution with a Pasteur pipette. Reaction mixture was allowed to continue stirring for a further 30 min. For the work up of reaction, 2 ml of concentrated HCl was added to a 20 ml ice-water solution. Reaction mixture was poured into this ice cold solution slowly with stirring. White solid product was collected as brown liquid. 4-Chlorobenzhydryl chloride and 4,4'-difluorobenzhydryl chloride were also synthesized from 4-chlorobenzhydryl chloride according to above procedure.

General procedure for preparation of benzhydrole and 4,4'-difluorobenzhydrole, respectively. Dichloromethane layer was washed with water again and extracted with water and ammonium chloride solution (10%), respectively. Dichloromethane layer was washed with water again and dried with anhydrous sodium sulfate. Solvent was evaporated under vacuum and solid product was recrystallized with ethanol/water.

N-sec-Butyl-4-(diphenylmethyl)piperazine-1-carboxamide (5a, CAS No: 1071382-92-7)

White, opaque, needle-shaped crystals, 68% (0.240 g), m.p. 198.4 ℃, UV (MeOH, λ_max, nm): 205 (log ε: 5.17), 224 (log ε: 4.69). FT-IR (KBr, cm⁻¹): 3342 (N–H), 3022 (C–H, aromatic), 2959 (C–H, aliphatic), 1619 (C=C, aromatic), 1540 (C=C, aromatic), 1426 (C–N). ¹H-NMR (DMSO, ppm): 0.78 (t, 3H, –CH₂–CH₃, J = 7.6 Hz); 0.98 (d, 3H, –CH₃, J = 6.8 Hz); 1.35 (m, 2H, –CH₂–CH₃); 2.23 (t, 4H, piperazine H₃, H₅, J = 4.6 Hz); 3.28 (t, 4H, piperazine H₂, H₆, J = 4.8 Hz); 3.53 (m, 1H, –NH–); 4.29 (s, 1H, (Ar₂)CH–); 6.02 (d, 1H, –CONH–, J = 7.6 Hz); 7.20 (m, 2H, diphenyl H₄, H₅); 7.30 (4H, diphenyl H₃, H₅, H₆, H₇, J = 7.6 Hz); 7.43 (4H, diphenyl H₂, H₆, H₇, H₈, J = 7.2 Hz). ¹³C-NMR (DMSO, ppm): 11.43 (C₆); 21.45 (C₂₂); 29.90 (C₂₀); 44.25 (C₁₄,1₆); 47.82 (C₁₉); 52.06 (C₁₅,1₇); 75.59 (C₇); 127.56 (C₁₄,1₆); 128.29 (C₁₉,₂₁,2₃); 129.20 (C₁₅,1₇,2₀,2₂); 143.30 (C₁₃,1₅); 157.67 (C₁₈, MS (m/z): 352.8 (M⁺); 253.7 ((C₇H₅)₂CH(N(CH₂)₂)NH⁺); 167.5 ((C₆H₅)₂CH⁺). Elementary analysis of C₂₂H₂₉N₃O (MW: 351.49 g/mol): C 75.18, H 8.32, N 11.96 (Calcd.); C 75.12, H 8.27, N 11.85 (Found).

N-tert-Butyl-4-(diphenylmethyl)piperazine-1-carboxamide (5b)

White, opaque, needle-shaped crystals, 62% (0.436 g), m.p. 192.4 ℃, UV (MeOH, λ_max, nm): 206 (log ε: 5.13), 227 (log ε: 4.62). FT-IR (KBr, cm⁻¹): 3322 (N–H), 3023 (C–H, aromatic), 2970 (C–H, aliphatic), 1621 (C=O, amide), 1536 (C=C, aromatic), 1260 (C–N). ¹H-NMR (DMSO, ppm): 1.22 (s, 9H, –(CH₃)₃); 2.23 (t, 4H, piperazine H₂, H₆, J = 4.8 Hz); 3.25 (t, 4H, diphenyl H₂, H₆, J = 4.4 Hz); 4.29 (s, 1H, (Ar₂)CH–); 5.68 (s, 1H, CONH); 7.19 (m, 2H, diphenyl H₃, H₅); 7.30 (4H, diphenyl H₃, H₅, H₆, H₇, J = 7.6 Hz); 7.43 (4H, diphenyl H₂, H₆, H₇, H₈, J = 7.2 Hz). Elemental analysis of C₂₂H₂₉N₃O (MW: 351.49 g/mol): C 75.18, H 8.32, N 11.96 (Calcd.); C 74.60, H 8.21, N 11.84 (Found).

N-Isopropyl-4-(diphenylmethyl)piperazine-1-carboxamide (5e)

White, opaque, clustered crystals, 94% (0.318 g), m.p. 220.4 ℃, UV (MeOH, λ_max, nm): 207 (log ε: 5.21), 227 (log ε: 4.81). FT-IR (KBr, cm⁻¹): 3367 (N–H), 3060 (C–H, aromatic), 2964 (C–H, aliphatic), 1611 (C=O, amide), 1538 (C=O, aromatic), 1254 (C–N). ¹H-NMR (DMSO, ppm): 0.98 (d, 6H, –CH(CH₃)₂, J = 6.8 Hz); 2.19 (t, 4H, piperazine H₂, H₆, J = 4.8 Hz); 3.25 (t, 4H, piperazine H₂, H₆, J = 5.2 Hz); 3.68 (m, 1H, –CH(CH₃)₂); 4.25 (s, 1H, (Ar₂)CH–); 6.05 (d, 1H, CONH, J = 7.6 Hz); 7.15 (m, 2H, diphenyl H₄, H₅); 7.26 (t, 4H, diphenyl H₂, H₆, H₇, H₈, J = 7.2 Hz); 7.39 (t, 4H, diphenyl H₂, H₆, H₇, H₈, J = 6.8 Hz). Elemental analysis of C₂₂H₂₉N₃O (MW: 374.46 g/mol): C 74.74, H 8.06, N 12.45 (Calcd.); C 74.89, H 7.73, N 12.30 (Found).

N-Ethyl-4-(diphenylmethyl)piperazine-1-carboxamide (5d)

White, shiny, flat crystals, 84% (0.294 g), m.p. 208.9 ℃, UV (MeOH, λ_max, nm): 203 (log ε: 5.11), 221 (log ε: 4.58). FT-IR (KBr, cm⁻¹): 3365 (N–H), 3024 (C–H, aromatic), 2978 (C–H, aliphatic), 1622 (C=O, amide), 1545 (C=C, aromatic), 1259 (C–N). ¹H-NMR (DMSO, ppm): 0.98 (t, 3H, –CH₃, J = 7.6 Hz); 2.23 (t, 4H, piperazine H₂, H₆, J = 4.8 Hz); 3.01 (m, 2H, –CH₂–); 3.28 (t, 4H, piperazine H₂, H₆, J = 5.2 Hz); 4.28 (s, 1H, (Ar₂)CH–); 6.41 (t, 1H, CONH, J = 5.2 Hz); 7.20 (m, 2H, diphenyl H₄, H₅); 7.29 (t, 4H, diphenyl H₂, H₆, H₇, H₈, J = 6.8 Hz).
N-sec-Butyl-4-[bis(4-fluorophenyl)methyl]piperazine-1-carboxamide (5i)

White, opaque, powdered crystals, 54% (0.208 g), m.p. 157.7°C. UV (MeOH, λmax nm): 207 (log ε: 5.24), 225 (log ε: 4.71). FT-IR (KBr, cm−1): 3310 (N–H), 3076 (C–H, aromatic), 2965 (C–H, aliphatic), 1615 (C=O, amide), 1548 (C=C, aromatic), 1247 (C–N), 1223 (C–F). 1H-NMR (DMSO, ppm): 0.8 (t, 3H, –CH3–CH3, J = 7.2 Hz); 0.98 (d, 3H, –CH–CH3, J = 6.8 Hz); 2.24 (t, 4H, piperazine H3, H5, J = 4.8 Hz); 2.5 (m, 2H, –CH–CH3); 3.28 (t, 4H, piperazine H3, H5, J = 4.8 Hz); 3.54 (m, 1H, –NH–CH2–); 4.38 (s, 1H, (Ar)–CH=); 6.04 (d, 1H, CONH, J = 7.6 Hz); 7.10–7.16 (m, 4H, diphenyl H2, H6, H2, H6); 7.41–7.45 (m, 4H, diphenyl H2, H6, H3, H5). MS (m/z): 388.95 (M+) 290.00 [(4-F-C6H4)2CH(N[C6H4]2)N+]; 203.55 (100%, (4-F-C6H4)CH+). Elemental analysis of C22H17F2N3O (MW: 387.46 g/mol); C 68.20, H 7.02, N 10.84 (Found); C 67.44, H 7.01, N 10.89 (Found).

N-tert-Butyl-4-[bis(4-fluorophenyl)methyl]piperazine-1-carboxamide (5j)

White, opaque, feather-like crystals, 82% (0.317 g), m.p. 162.4°C. UV (MeOH, λmax nm): 208 (log ε: 5.32), 227 (log ε: 4.78). FT-IR (KBr, cm−1): 3332 (N–H), 3046 (C–H, aromatic), 2968 (C–H, aliphatic, 1623 (C=O, amide), 1537 (C=C, aromatic), 1259 (C–N), 1219 (C–F). 1H-NMR (DMSO, ppm): 1.22 (s, 9H, C(CH3)3); 2.20 (t, 4H, piperazine H3, H5, J = 4.8 Hz); 3.24 (t, 4H, piperazine H3, H5, J = 4.8 Hz); 4.38 (s, 1H, (Ar)–CH=); 5.68 (s, 1H, CONH); 7.10–7.16 (m, 4H, diphenyl H2, H6, H2, H6); 7.41–7.45 (m, 4H, diphenyl H2, H6, H3, H5). MS (m/z): 388.88 (100%, M+) 203.51 [(4-F-C6H4)CH+]. Elemental analysis of C22H17F2N3O (MW: 387.46 g/mol); C 68.20, H 7.02, N 10.84 (Found); C 67.96, H 7.32, N 10.87 (Found).

N-Butyl-4-[bis(4-fluorophenyl)methyl]piperazine-1-carboxamide (5k)

White, opaque, flat crystals, 45% (0.174 g), m.p. 132.9°C. UV (MeOH, λmax nm): 209 (log ε: 5.43), 226 (log ε: 4.83). FT-IR (KBr, cm−1): 3402 (N–H), 3073 (C–H, aromatic), 2962 (C–H, aliphatic, 1629 (C=O, amide), 1531 (C=C, aromatic), 1251 (C–N), 1217 (C–F). 1H-NMR (DMSO, ppm): 0.85 (t, 3H, –CH3, J = 7.2 Hz) 1.20–1.27 (m, 2H, –CH–CH3); 1.31–1.37 (m, 4H, –CH–CH–CH3); 2.21 (t, 4H, piperazine H3, H5, J = 4.4 Hz); 2.95–3.06 (q, 2H, –O–CH2–); 3.37 (t, 4H, piperazine H3, H5, J = 5.2 Hz); 4.38 (s, 1H, (Ar)–CH=); 6.38 (t, 1H, CONH); 7.15–7.15 (m, 4H, diphenyl H2, H6, H2, H6); 7.41–7.45 (m, 4H, diphenyl H2, H6, H3, H5). MS (m/z): 388.93 (100%, M+) 203.55 [(4-F-C6H4)CH+]. Elemental analysis of C22H17F2N3O (MW: 387.46 g/mol); C 68.20, H 7.02, N 10.84 (Found); C 67.92, H 6.82, N 10.85 (Found).

N-Ethyl-4-[bis(4-fluorophenyl)methyl]piperazine-1-carboxamide (5l)

White, white, flat crystals, 96% (0.323 g), m.p. 213.6°C. UV (MeOH, λmax nm): 207 (log ε: 5.32), 226 (log ε: 4.75). FT-IR (KBr, cm−1): 3343 (N–H), 3026 (C–H, aromatic, 2954 (C–H, aliphatic), 1625 (C=O, amide), 1546 (C=C, aromatic), 1255 (C–N). 1H-NMR (DMSO, ppm): 2.23 (t, 4H, piperazine H3, H5, J = 4.8 Hz); 3.30 (t, 4H, piperazine H3, H5, J = 4.8 Hz); 3.68 (t, 2H, –CH2–CH2–, J = 5.2 Hz); 4.29 (s, 1H, (Ar)–CH=); 5.0 (dd, 2H, –CH–CH3, J1 = 17.2 Hz, J2 = 8 Hz, J3 = 1.6 Hz); 5.78 (m, 1H, –CH=CH2); 6.61 (t, 1H, CONH, J = 5.2 Hz); 7.17 (t, 2H, diphenyl H2, H6, J = 7.6 Hz); 7.29 (t, 4H, diphenyl H2, H6, H3, H5, J = 7.6 Hz); 7.43 (d, 4H, diphenyl H2, H6, H3, H5, J = 8.8 Hz). Elemental analysis of C22H17F2N3O (MW: 381.47 g/mol); C 69.27, H 7.13, N 11.02 (Calcd.); C 69.24, H 6.96, N 10.96 (Found).

N-tert-Butyl-4-[bis(4-fluorophenyl)methyl]piperazine-1-carboxamide (5m)

White, opaque, feather-like crystals, 82% (0.317 g), m.p. 162.4°C. UV (MeOH, λmax nm): 208 (log ε: 5.32), 227 (log ε: 4.78). FT-IR (KBr, cm−1): 3332 (N–H), 3046 (C–H, aromatic), 2968 (C–H, aliphatic, 1623 (C=O, amide), 1537 (C=C, aromatic), 1259 (C–N), 1219 (C–F). 1H-NMR (DMSO, ppm): 1.22 (s, 9H, C(CH3)3); 2.20 (t, 4H, piperazine H3, H5, J = 4.8 Hz); 3.24 (t, 4H, piperazine H3, H5, J = 4.8 Hz); 4.38 (s, 1H, (Ar)–CH=); 5.68 (s, 1H, CONH); 7.10–7.16 (m, 4H, diphenyl H2, H6, H2, H6); 7.41–7.45 (m, 4H, diphenyl H2, H6, H3, H5). MS (m/z): 388.88 (100%, M+) 203.51 [(4-F-C6H4)CH+]. Elemental analysis of C22H17F2N3O (MW: 387.46 g/mol); C 68.20, H 7.02, N 10.84 (Calcd.); C 67.92, H 6.82, N 10.85 (Found).
N-Isopropyl-4-[bis(4-fluorophenyl)methyl]piperazine-1-carboxamide (5m)

White, opaque, powdercd crystals, 92% (0.345 g), m.p. 169.9°C. UV (MeOH, 360 nm): 205 (log ε: 5.25), 223 (log ε: 4.47). FT-IR (KBr, cm⁻¹): 3331 (N-H, aromatic), 3074 (C-H, aliphatic), 2974 (C-H, aliphatic), 1615 (C=O, amide), 1547 (C=C, aromatic), 1252 (C-N), 1215 (C-F). ¹H-NMR (DMSO, ppm): 1.01 (s, 3H, (Ar)2CH–), 1.17 (s, 9H, –CH(CH3)3); 2.22 (t, 4H, piperazine H3,H 5, J=4.4 Hz); 3.19–3.22 (m, 4H, piperazine H2,H 6, J=5.6 Hz); 7.11–7.15 (m, 4H, diphenyl H2,H 6, H3,H 5); 7.42–7.47 (m, 4H, diphenyl H1, H2, H3, H4). MS (m/z): 374.87 (M⁺), 289.72 [(4-F-C6H4)2CH(N(CH2)2NH)⁺]; 203.54 (100%, (4-F-C6H4)2CH⁺). Elemental analysis of C21H25F2N3O (MW: 373.44 g/mol); C 67.54, H 6.75, N 11.25 (Calcd.). C 67.87, H 6.64, N 11.20 (Found).

Ethyl 2-[bis(4-fluorophenyl) methyl]piperazinocarbamate lactate (5a)

White, opaque, powdercd crystals, 20% (0.08 g), m.p. 152.3°C. UV (MeOH, λmax nm): 203 (log ε: 4.89), 221 (log ε: 4.29). FT-IR (KBr, cm⁻¹): 3359 (N-H, aromatic), 3070 (C-H, aromatic), 2978 (C-H, aliphatic), 1748 (C=O, ester), 1640 (C=O, amide), 1602 (C=C, aromatic), 1224 (C-O), 1198 (C-N), 1153 (C-F). ¹H-NMR (DMSO, ppm): 1.17 (t, 3H, –CH3, J=7.2 Hz); 2.23 (t, 4H, piperazine H3,H 5, J=5.2 Hz); 3.11 (t, 4H, piperazine H2,H 6, J=4.8 Hz); 3.68 (d, 2H, –NH–CH2–, J=6.0 Hz); 4.03–4.08 (q, 2H, –O–CH2–); 4.39 (s, 1H, (Ar)CH–); 6.93 (t, 1H, CONH, J=6.0 Hz); 7.11–7.16 (m, 4H, diphenyl H2,H 6, H3,H 5); 7.42–7.46 (m, 4H, diphenyl H1, H2, H3, H4). Elemental analysis of C22H28ClN3O (MW: 385.93 g/mol); C 63.46, H 6.05, N 10.02 (Calcd.). C 63.34, H 6.55, N 10.02 (Found).

N-(4-Bromophenyl)-4-[bis(4-fluorophenyl)methyl]piperazine-1-carboxamide (5o)

White, opaque, powdercd crystals, 67% (0.325 g), m.p. 210.9°C. UV (MeOH, λmax nm): 202 (log ε: 4.31), 237 (log ε: 4.15), 246 (log ε: 4.12). FT-IR (KBr, cm⁻¹): 3290 (N-H, aromatic), 3044 (C-H, aromatic), 2999 (C=O, aliphatic), 1646 (C=O, amide), 1506 (C=C, aromatic), 1246 (C-N), 1224 (C-F). ¹H-NMR (DMSO, ppm): 2.29 (t, 4H, piperazine H3,H 5, J=4.4 Hz); 3.45 (t, 4H, piperazine H2,H 6, J=4.8 Hz); 4.44 (s, 1H, (Ar)CH–); 7.12–7.47 (m, 12H, aromatic). MS (m/z): 618.81 (M⁺). Elemental analysis of C22H23BrClN3O (MW: 484.82 g/mol); C 52.97, H 4.38, N 6.84 (Calcd.). C 52.92, H 4.38, N 8.73 (Found).

N-sec-Butyl-4-[4-(chlorophenyl)phenyl]methyl)piperazine-1-carboxamide (5p)

White, shiny, clustered crystals, 17% (0.06 g), m.p. 288.6°C. UV (MeOH, λmax nm): 205 (log ε: 5.24), 224 (log ε: 4.46). FT-IR (KBr, cm⁻¹): 3363 (N-H, aromatic), 3070 (C-H, aromatic), 2978 (C-H, aliphatic), 1620 (C=O, amide), 1539 (C=C, aromatic), 1254 (C-N), 1090 (C-Cl). ¹H-NMR (DMSO, ppm): 0.98 (t, 3H, –CH3, J=7.2 Hz); 2.22 (t, 4H, piperazine H3,H 5, J=4.4 Hz); 3.00–3.03 (m, 2H, –CH2–); 3.27 (t, 4H, piperazine H2,H 6, J=5.2 Hz); 4.34 (s, 1H, (Ar)CH–); 6.41 (t, 1H, CONH, J=5.6 Hz); 7.18–7.46 (m, 9H, aromatic), 7.90 (d, 1H, diphenyl). Elemental analysis of C23H25ClN3O (MW: 375.88 g/mol); C 67.12, H 6.76, N 11.74 (Calcd.). C 67.22, H 6.69, N 11.79 (Found).

N-(4-Chlorophenyl)-4-[bis(4-chlorophenyl)methyl]piperazine-1-carboxamide (5q)

White, shiny, flat crystals, 36% (0.137 g), m.p. 190.3°C. UV (MeOH, λmax nm): 207 (log ε: 5.29), 225 (log ε: 4.52). FT-IR (KBr, cm⁻¹): 3371 (N-H, aromatic), 2968 (C-H, aliphatic), 1629 (C=O, amide), 1538 (C=C, aromatic), 1257 (C-N), 1092 (C-Cl). ¹H-NMR (DMSO, ppm): 1.19 (s, 9H, –(CH2)3); 2.19 (t, 4H, piperazine H3,H 5, J=4.8 Hz); 3.21 (t, 4H, piperazine H2,H 6, J=4.8 Hz); 4.31 (s, 1H, (Ar)CH–); 5.65 (s, 1H, CONH); 7.17–7.42 (m, 9H, diphenyl). Elemental analysis of C24H20Cl2N3O (MW: 389.51 g/mol); C 68.47, H 7.31, N 10.89 (Calcd.). C 68.65, H 7.20, N 10.93 (Found).
N-(2-Phenylethyl)-4-[(4-chlorophenyl)phenyl]methyl piperazine-1-carbothioamide (5v)

White, opaque, feather-like crystals, 49% (0.212 g), m.p. 147.8°C. UV (MeOH, λ_{max}, nm): 205 (log ε: 4.52), 245 (log ε: 4.07). FT-IR (KBr, cm⁻¹): 3307 (N-H, amide), 1591 (C=N, aromatic), 1254 (C-N), 1089 (C-Cl). ¹H-NMR (DMSO, ppm): 2.13 (t, 4H, piperazine H₂, H₆, J = 4.8 Hz); 3.46 (t, 4H, piperazine H₂, H₆, J = 4.4 Hz); 4.41 (s, 1H, (Ar)CH-); 7.21–7.49 (m, 12H, aromatic H's); 8.37 (s, 1H, CONH). Elemental analysis of C₂₃H₂₃Cl₂N₃O₄: MW: 474.81 g/mol; C 60.71, H 4.67, N 8.85 (Calcd.); C 60.70, H 4.77, N 9.18 (Found).

N-(2-Benzyloxymethyl)-4-[(4-chlorophenyl)phenyl]methyl piperazine-1-carboxamide (5w)

White, opaque, feather-like crystals, 49% (0.212 g), m.p. 147.8°C. UV (MeOH, λ_{max}, nm): 205 (log ε: 4.52), 245 (log ε: 4.07). FT-IR (KBr, cm⁻¹): 3307 (N-H, amide), 1591 (C=N, aromatic), 1254 (C-N), 1089 (C-Cl). ¹H-NMR (DMSO, ppm): 2.22 (t, 4H, piperazine H₂, H₆, J = 4.4 Hz); 2.69 (t, 2H, –CH₂C₂H₅, J = 6.8 Hz); 3.10 (q, 2H, –NHCH₂–); 2.38 (t, 4H, piperazine H₂, H₆, J = 5.2 Hz); 4.34 (s, 1H, (Ar)CH-); 6.55 (t, 1H, CONH, J = 5.6 Hz); 7.15–7.46 (m, 14H, aromatic H's). Elementary analysis of C₂₃H₂₃Cl₂N₃O₄: MW: 433.97 g/mol; C 71.96, H 6.50, N 9.68 (Calcd.); C 72.04, H 6.72, N 9.70 (Found).

N-(4-Bromomethyl)-4-[(4-chlorophenyl)phenyl]methyl piperazine-1-carboxamide (5x)

Yellowish white, opaque, powdered crystals, 14% (0.066 g), m.p. 176.8°C. UV (MeOH, λ_{max}, nm): 205 (log ε: 4.23), 223 (log ε: 4.11). FT-IR (KBr, cm⁻¹): 3258 (N-H, amide), 2972 (C-H, aliphatic), 1606 (C=C, aromatic), 1236 (C=S, thioamide), 1189 (C-F). ¹H-NMR (DMSO, ppm): 1.45 (s, 9H, –(CH₃)₃); 2.96–3.15 (m, 4H, piperazine H₂, H₆); 3.65 (t, 4H, piperazine H₂, H₆); 4.59 (d, 1H, (Ar)CH-, J = 14.4 Hz); 5.75 (d, 1H, CNH, J = 8.8 Hz); 7.17–7.33 (m, 4H, diphenyl H₂, H₆, H₈, H₉); 7.95 (bs, 4H, diphenyl H₂, H₆, H₈, H₉); 12.25 (bs, 1H, N-H salt). MS (m/z): 404.90 (100%, M⁺ – Cl); 205.3 (4-F-C₆H₄), CH⁺). Elemental analysis of C₂₃H₂₂Cl₂F₂N₂S: MW: 439.99 g/mol; C 60.05, H 6.41, N 9.55, S 7.29 (Calcd.); C 59.55, H 6.45, N 9.47, S 6.64 (Found).

N-Cyclohexyl-4-[(4-fluorophenyl)phenyl]methyl piperazine-1-carbothioamide hydrochloride (6a)

White, shiny, needle-shaped crystals, 50% (0.214 g), m.p. 198.2°C. UV (MeOH, λ_{max}, nm): 202 (log ε: 4.10), 224 (log ε: 4.02). 248 (log ε: 3.88). FT-IR (KBr, cm⁻¹): 3328 (N-H, amide), 3060 (C-H, aromatic), 2996 (C-H, aliphatic), 1603 (C=C, aromatic), 1299 (C-N), 1221 (C-S, thioamide), 1104 (C-F). ¹H-NMR (DMSO, ppm): 1.13–1.21 (m, 5H, cyclohexyl); 1.53–1.79 (m, 6H, cyclohexyl); 2.22 (t, 4H, piperazine H₂, H₆, J = 4.8 Hz); 3.71 (t, 4H, piperazine H₂, H₆, J = 4.8 Hz); 4.12 (s, 1H, CNH); 4.40 (s, 1H, (Ar)CH-); 7.09–7.14 (m, 4H, diphenyl H₂, H₆, H₈, H₉); 7.39–7.43 (m, 4H, diphenyl H₂, H₈, H₉); 1.33–1.46 (m, 5H); 7.17–7.33 (m, 4H, diphenyl H₂, H₆, H₈, H₉). ¹C-NMR (DMSO, ppm): 14.26 (C₂H₅); 25.18 (C₂H₅); 31.97 (C₂H₅); 47.06 (C₂H₅); 50.85 (C₂H₅); 54.28 (C₂H₅); 72.32 (C₂H₅); 115.14 (C₂H₅); 112.55 (C₂H₅); 129.35 (C₂H₅); 129.43 (C₂H₅); 132.82 (C₂H₅); 138.15 (C₂H₅); 139.79 (C₂H₅); 161.22 (C₂H₅); 180.14 (C₂H₅). MS (m/z): 430.95 (100%, M⁺); 203.65 (4-F-C₆H₄), CH⁺). Elemental analysis of C₂₃H₂₂F₂N₂S: MW: 429.57 g/mol; C 67.10, H 6.80, N 9.78, S 7.46 (Calcd.); C 66.94, H 6.94, N 9.89, S 7.42 (Found).

N-Ethyl-4-[(4-chlorophenyl)phenyl]methyl piperazine-1-carbothioamide (6c)

White, opaque, powdered crystals, 15% (0.056 g), m.p. 150.6°C. UV (MeOH, λ_{max}, nm): 204 (log ε: 4.25), 225 (log ε: 4.13). FT-IR (KBr, cm⁻¹): 3294 (N-H, amide), 2966
N-Isopropyl-4-[4-(chlorophenyl)phenyl]methyl)piperazine-1-carbothioamide (6d)

White, shiny, needle-shaped crystals, 39% (0.15 g), m.p. 252.4°C. UV (MeOH, λ_max nm): 203 (log ε: 4.23), 223 (log ε: 4.15). FT-IR (KBr, cm⁻¹): 3371 (N-H), 3059 (C-H, aromatic), 2967 (C-H, aliphatic), 1539 (C=C, aromatic), 1270 (C-N), 1232 (C=S, thioamide), 1232 (C-N), 1001 (C-Cl). ¹H-NMR (DMSO, ppm): 1.09 (d, 6H, –(CH₃)₂, J 6.8 Hz); 2.27 (t, 4H, piperazine H₃,H₅, J 4.8 Hz); 3.76 (t, 4H, piperazine H₂,H₆, J 4.8 Hz); 4.39 (s, 1H, (Ar)₂CH–); 4.79 (d, 2H, –CH₂–, J 3.8 Hz); 5.80–5.90 (m, 1H, –CH); 7.19–7.46 (m, 9H, diphenyl); 7.61 (t, 1H, CSNH). Elemental analysis of C₂₀H₂₃ClN₃S (MW: 373.97 g/mol); C 64.34, H 6.49, N 10.83, S 8.29 (Found).

N-Allyl-4-[4-(chlorophenyl)phenyl]methyl)piperazine-1-carbothioamide (6e)

White, opaque, powder crystals, 10% (0.040 g), m.p. 139.4°C. UV (MeOH, λ_max nm): 204 (log ε: 4.27), 225 (log ε: 4.13). FT-IR (KBr, cm⁻¹): 3296 (N-H), 3023 (C-H, aromatic), 2960 (C-H, aliphatic), 1528 (C=C, aromatic), 1252 (C-N), 1224 (C=S, thioamide), 1223 (C-N), 1090 (C-Cl). ¹H-NMR (DMSO, ppm): 2.28 (t, 4H, piperazine H₃,H₅, J 5.2 Hz); 3.79 (t, 4H, piperazine H₂,H₆, J 4.8 Hz); 4.15 (t, 2H, –CH₂–, J 6.5 Hz); 4.39 (s, 1H, (Ar)₂CH–); 5.01–5.11 (dd, 2H, –CH₂–, J₁ = 7.2 Hz, J₂ = 8.6 Hz, J₃ = 1.6 Hz); 5.80–5.90 (m, 1H, –CH₂–, J = 7.19–7.46 (m, 9H, diphenyl); 7.80 (t, 1H, CSNH). Elementary analysis of C₂₁H₂₆ClN₃S (MW: 387.97 g/mol); C 65.01, H 6.75, N 10.83, S 8.29 (Found).

N-Benzyl-4-[4-(chlorophenyl)phenyl]methyl)piperazine-1-carbothioamide hydrochloride (6g)

White, opaque, featherlike crystals, 20% (0.080 g), m.p. 125.5°C. UV (MeOH, λ_max nm): 204 (log ε: 4.43), 227 (log ε: 4.35). FT-IR (KBr, cm⁻¹): 3261 (N-H), 3028 (C-H, aromatic), 2958 (C-H, aliphatic), 1541 (C=C, aromatic), 1298 (C-N), 1201 (C=S, thioamide), 1201 (C-N), 1001 (C-Cl). ¹H-NMR (DMSO, ppm): 0.87 (t, 3H, –CH₃CH₂CH₃, J = 7.2 Hz); 1.20–1.29 (m, 2H, –CH₂CH₂CH₃); 1.44–1.52 (m, 2H, –CH₂CH₂CH₃); 2.27 (t, 4H, piperazine H₃,H₅, J = 4.8 Hz); 3.42–3.47 (q, 2H, –NHCH₃); 3.75 (t, 4H, piperazine H₂,H₆, J = 4.8 Hz); 4.39 (s, 1H, (Ar)₂CH–); 7.19–7.46 (m, 9H, diphenyl); 7.58 (t, 1H, CSNH), J = 5.6 Hz). Elemental analysis of C₂₂H₂₅ClN₃S (MW: 401.17 g/mol); C 65.73, H 7.02, N 10.45, S 7.98 (Found); C 66.06, H 7.07, N 10.56, S 8.05 (Found).

Cytotoxicity studies

The cytotoxic activity of the synthesized compounds was investigated initially on liver (HUH-7), breast (MCF-7) and colo (HCT-116) cancer cell lines, by means of SRB assays in triplicate. Serial dilutions from 100 μM to 2.5 μM were used, 5-fluorouracil (5-FU) was the reference compound and camptothecin (CPT) was the positive control for the cytotoxic effect.

Cell culture

The human cancer cell lines were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. Each cell line was maintained in an incubator at 37°C supplied with 5% CO₂ and 95% air.

NCl-60 SRB assay

Cancer cells (range of 2000 cell/well to 5000 cell/well) were inoculated into 96-well plates in 200 μl of media and incubated in 37°C incubators containing 5% CO₂ and 95% air. After a 24 h incubation period, one plate for each cell line was fixed with 100 μl of 10% ice-cold trichloroacetic acid (TCA). This plate represents the behavior of the cells just prior to the drug treatment and is accepted as the time-zero plate. The compounds to be tested were solubilized in DMSO to a final concentration of 40 mM and stored at +4°C. While treating the cells with the compounds, the corresponding volume of the compound was applied to the cell to achieve the desired drug concentration and diluted through serial dilution. After the drug treatment, the cells were incubated in 37°C incubators containing 5% CO₂ and 95% air for 72 h. Following the termination of the incubation period after the drug treatment, the cells were fixed with 100 μl of 10% ice-cold TCA and incubated in the dark at +4°C for 1 h. Then the TCA was washed away with ddH₂O five times and the plates were left to air dry. For the final step, the plates were stained with 100 μl of 0.4% SRB solution in 1% acetic acid solution. Following staining, the plates were incubated in dark for 10 min at room temperature. The unbound dye was washed away using 1% acetic acid and the plates were left to air dry. To measure the absorbance results, the bound stain was then solubilized using 200 μl of 10 mM Tris-Base. The OD values were obtained at 515 nm.

Results and discussion

Chemistry

The synthesis of the benzhydryl piperazine derivatives (5a–y) and (6a–g) is outlined in Figure 1. Reduction with sodium borohydride of benzophenone, 4-chlorobenzophenone and 4,4'-difluorobenzophenone afforded benzhydryl derivatives which were chlorinated with HCl and anhydrous calcium chloride. Resulting benzhydryl chloride derivatives were used for N-alkylation of piperazine to give 1-benzhydryl piperazine, 4-chlorobenzhydryl piperazine and 4,4'-difluorobenzhydryl piperazine. The final step was nucleophilic addition to isocyanates or isothiocyanates in order to obtain benzhydrylpiperazine derivatives (5a–y) and (6a–g).
Synthesized compounds were identified with IR, UV and 1H-NMR spectra. In addition, some compounds were selected for LC-MS and 13C-NMR spectral evaluation. In UV spectra of carboxamide derivatives there are two significant bands at 205 and 224 nm, which represent \( \pi \rightarrow \pi^* \) and \( n \rightarrow \pi^* \) transitions. In UV spectra of thioamide derivatives there are three significant bands nearly at 202, 224 and 248 nm, which represent \( \pi \rightarrow \pi^* \) and \( n \rightarrow \pi^* \) transitions. In IR spectrum of carboxamide derivatives, characteristic N–H stretching band is observed nearly at 3332 cm\(^{-1}\). Other stretching bands are observed approximately at 3020 cm\(^{-1}\) (C–H; aromatic), 2965 cm\(^{-1}\) (C–H; aliphatic), 1625 cm\(^{-1}\) (C=O; amide), 1520 cm\(^{-1}\) (C=C; aromatic) and 1250 cm\(^{-1}\) (C–N). In IR spectrum of thioamide derivatives, characteristic N–H stretching band is observed nearly at 3330 cm\(^{-1}\). Other stretching bands are observed approximately at 3060 cm\(^{-1}\) (C–H; aromatic), 2995 cm\(^{-1}\) (C–H; aliphatic), 1600 cm\(^{-1}\) (C=C; aromatic), 1300 cm\(^{-1}\) (C=N) and 1220 cm\(^{-1}\) (C=S) and 1100 cm\(^{-1}\) (C=F). In H\(^1\)-NMR spectra of carboxamide derivatives the protons of piperazine are seen approximately at 2.23 and 3.36 ppm as broad singlets. Diphenylmethyl C–H gives a singlet nearly at 4.5 ppm. Protons of aromatic rings give multiplets at 7–7.5 ppm. Thioamide N–H is observed approximately at 7.5 ppm. The 13C-NMR spectrum of 5a shows characteristic peaks of the carboxamide derivatives approximately at 45 and 50 ppm for piperazine ring, 75 ppm for diphenylmethyl carbon and 150 ppm for carbonyl group. The 13C-NMR spectrum of 6b shows characteristic peaks of the thioamide derivatives nearly at 45 and 50 ppm for piperazine ring, 70 ppm for diphenylmethyl carbon and 180 ppm for thio carbonyl group.

Cytotoxicity
The cytotoxic activity of the synthesized compounds 5a–y and 6a–g was investigated on liver (HUH-7), breast (MCF-7) and colon (HCT-116) cancer cell lines, by means of SRB assays in triplicate. As shown in Table 2, all tested compounds were screened with mean 50% growth inhibition concentration (GI\(_{50}\)) in micromolar concentration range.

Most of the nonsubstituted benzhydryl piperazine derivatives are inactive or they have low activities against all cancer...
cell lines. It should also be noted that, in general, 4-chlorobenzhydroxylpiperazinyl derivatives have higher activities becoming superior over their 4,4′-difluoro and nonsubstituted counterparts. Moreover, thioamide derivatives are more potent than carboxamide derivatives against all cancer cell lines. Corresponding compound groups representing these findings are detailed in Table 3.

Compounds 5c, 5m, 5s and 6d have the same substituents on NH group (R3 = iso-propyl). Compound 5c has no cytotoxicity against any of these cancer cell lines. However, 5m has slight cytotoxicity, 5s has good cytotoxicity and 6d has the highest cytotoxicity against all three cancer cell lines.

Compounds 5e and 5u have the same substituents on NH group (R3 = 2,6-dichlorophenyl). 5e has no cytotoxicity against any of the cancer cell lines. Interestingly, 5u has increased cytotoxicity against all the cancer cell lines.

Compounds 5f and 5x have the same substituents on NH group (R3 = 2-benzylphenyl). 5f has no cytotoxicity against none of these cancer cell lines. However, 5x has good cytotoxicity against all the cancer cell lines.

Compounds 5h, 5t and 6e have the same substituents on NH group (R3 = allyl). 5h has no cytotoxicity against HUH-7 and HCT-116 cell lines nevertheless it has good cytotoxicity against MCF-7 cell line. However, 5t has elevated cytotoxicity and 6e has the highest cytotoxicity against all three cancer cell lines.

In general, nonsubstituted benzhydryl derivatives are inactive or have low inhibition whereas 4-chlorobenzhydryl derivatives are more active than other compounds against HUH-7 cell line.

The most active compounds against HUH-7 cell line are 5y (GI50 = 1.29 μM) and 6a (GI50 = 5.97 μM). Additionally, most of the compounds have higher cytotoxicity against HUH-7 than reference compound 5-fluorouracil.

Among the carboxamide derivatives, compounds bearing electron withdrawing substituents on phenyl ring such as 5o (GI50 = 9.46 μM), 5u (GI50 = 6.44 μM), 5w (GI50 = 8.54 μM) and 5y (GI50 = 1.29 μM) are highly active against HUH-7 cell line. In addition, alkyl substituted derivatives, except thioamide derivatives, have no (5a-d, 5h, 5n) or low inhibition (5i-m, 5p-t).

Thioamide derivatives are generally cytotoxic against HUH-7 cell line. It can be noted that thioamides show higher activity than their carboxamide derivatives, which can be exemplified by compounds 5j (GI50 = 29.96 μM) compared with 6a (GI50 = 5.97 μM), 5r (GI50 = 20.92 μM) compared with 6c (GI50 = 10.81 μM), 5s (GI50 = 15.36 μM) compared with 6d (GI50 = 6.20 μM) and 5t (GI50 = 16.29 μM) compared with 6e (GI50 = 9.95 μM).
The most active compounds against MCF-7 cell line are 5u (GI50 = 6.14 μM) and 6e (GI50 = 4.94 μM). Furthermore, we observed that compound 6e was less toxic in MCF-12A (GI50 = 8.5 μM), which is a normal-like breast epithelial cell line (Table 4).

Against MCF-7 cell line, nonsubstituted benzhydryl carboxamide derivatives (except 5d and 5h) and 5i, 5j, 5l, 6b, 6e show no inhibition. Alkyl-substituted carboxamide derivatives have low activity values such as 5d (GI50 = 25.7 μM), 5k (GI50 = 19.03 μM), 5m (GI50 = 45.23 μM), 5n (GI50 = 36.14 μM), 5r (GI50 = 60.24 μM). However, compounds such as 5u (GI50 = 6.14 μM) and 5y (GI50 = 6.34 μM) that contain phenyl ring with electron withdrawing substituents are highly cytotoxic.

Against HCT-116 cell line, 5b (GI50 = 1.01 μM) and 5y (GI50 = 1.81 μM) are the most active derivatives. In addition, most of the compounds have higher cytotoxicity against HCT-116 than reference compound 5-fluorouracil.

With the exception of 5b, nonsubstituted benzhydryl carboxamide derivatives present no inhibition against HCT-116 cell line. 4-Chlorobenzhydryl carboxamide derivatives are higher in activity than 4,4′-difluorobenzhydryl carboxamide derivatives demonstrated with compounds 5i (GI50 = 24.48 μM) and 5p (GI50 = 9.33 μM) or compounds 5j (GI50 = 28.4 μM) and 5q (GI50 = 9.33 μM). Thioamides generally show good activity values considering HCT-116 cell line.

### Conclusion

In this study, 32 benzhydrylpiperazine derivatives with carboxamide and thioamide moieties were prepared. *In vitro* cytotoxic activities were screened against hepatocellular (HUH-7), breast (MCF-7) and colorectal (HCT-116) cancer cell lines by SRB assay. Most of the compounds presented higher cytotoxicity against HUH-7 and HCT-116 cancer cell lines in comparison with reference compound 5-fluorouracil. Interestingly, 4-chlorobenzhydrylpiperazine derivatives were more active than benzhydrylpiperazine and 4,4′-difluorobenzhydrylpiperazine derivatives. In addition, thioamide derivatives were observed to have markedly elevated cytotoxicity values opposed to their carboxamide analogs. Future synthesis of similar derivatives will take place to create a larger set of compounds, in order to produce a rational quantitative structure-activity relationship (QSAR) mapping. Since 4-chlorobenzhydrylpiperazine derivatives are chiral compounds, further exploration of chiral separation methods will be performed. The primary ambition regarding future research is to evaluate the mechanism of cytotoxicity.

### Declaration of interest

The authors have declared no conflicts of interest.

### References


