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Research Article

4-amidobenzhydryl analogue as stable acetoxychavicol acetate (ACA): Synthesis, characterisation and cytotoxic activity against human myeloid leukaemia cell lines (KASUMI-1)

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Abstract
A series of 4-amidobenzhydryl-type analogues, designed as mimics of the stable compound 1′-acetoxychavicol acetate (ACA), were successfully synthesised by acylation reaction of (4-aminophenyl)(phenyl)methanol with various acyl chlorides. All the synthesised analogues were fully characterised by comprehensive spectroscopic techniques. The cytotoxic activity of these analogues was evaluated using the MTT assay against the human myeloid leukemia cell line Kasumi-1. The structure-activity relationship (SAR) analysis was conducted to examine the impact of different functional groups on the cytotoxic properties of the compounds. Compound **3a** exhibit the highest cytotoxic activity with IC_{50} values of 247.35 µM (24h), 106.01 µM (48h) and 98.94 µM (72h). SAR analysis indicated that aliphatic substitutions at the carboxamide and ester positions enhanced cytotoxic activity, while phenyl and cyclohexyl substitutions resulted in decreased activity. These results indicate that 4-amidobenzhydryl analogues, in particular compound **3a**, are promising anticancer agents, with compound **3a** emerging as a lead candidate for further development.

Keywords: 4-amidobenzhydryl-type analogues, 1′-acetoxychavicol acetate, cytotoxic, MTT assay, human myeloid leukemia

Introduction

Cancer continues to be a major cause of morbidity and mortality around the world, necessitating the constant search for novel therapeutic agents with improved efficacy and selectivity. Among the diverse natural products investigated, 1′-acetoxychavicol acetate (ACA), a compound derived from the rhizomes of Alpinia conchigera and Alpinia galanga, has garnered significant attention due to its potent anticancer activity against various cancer cell lines, including human myeloid leukemia cells [1-7]. Our previous study on ACA showed that this compound has extensive potential to inhibit MDA-MB-231 (4.8 µM), HSC-2 (5.0 µM), HSC-4 (5.5 µM), EJ-28 (8.2 µM), RT-112 (14.1 µM), CaSki (17.0 µM), HepG2 (18.0 µM), PC-3 (26.7 µM) and MCF-7 (30.0 µM) [1-3]. Our previous study has shown that ACA exerts its cytotoxic effect via multiple mechanisms, including induction of apoptosis through dysregulation of nuclear factor- kappaB (NF-κB) and suppression of IKKα/β activation [8-9]. However, its inherent chemical instability in serum, poor solubility in water and limited bioavailability have posed significant challenges for its clinical application [10-12]. In addition, ACA is also unstable in a 5% ethanol–aqueous solution and transforms into other by-products, which do not show cytotoxic effects via [3,3]-sigmatropic rearrangement or hydrolysis [13-14].

To overcome these limitations, structural modification of ACA has been explored as a strategy to improve its stability and therapeutic potential. In this context, 4-amidobenzhydryl-type analogues offer a promising scaffold for the development of more stable ACA derivatives. The incorporation of an amide functional group into such analogues not only improves their chemical stability, but also provides opportunities for fine-tuning their physicochemical and biological properties. Previous studies on benzhydryl-based compounds have demonstrated their potential as anticancer agents, emphasising their role as promising candidates for further development [14-15].

In this study, we report the synthesis and characterisation of 4-amidobenzhydryl-type analogues designed as stable ACA mimics. The cytotoxic activity of these analogues was evaluated against human myeloid leukemia cell lines (Kasumi-1) to assess their potential as anticancer agents. In addition, a detailed structural analysis was performed to elucidate structure-activity relationships, providing valuable insights into the design of future derivatives with enhanced anticancer efficacy. By addressing the limitations of ACA through rational structural modifications, this work contributes to the development of novel chemotherapeutic agents with improved stability and efficacy. This research also emphasises the importance of natural product-inspired design in the search for new anticancer compounds.

Materials and Methods

Generals

All reactions were carried out in heat-dried glassware under an atmosphere of nitrogen unless otherwise stated. All liquid transfers were conducted using standard syringe or cannula techniques. DCM was dried under molecular sieves 4Å. All other reagents were obtained from Merck or Aldrich and used as received. Column chromatography was performed on silica gel (Merck, 60 Å C. C. 40-63 mm) as the stationary phase. Thin Layer Chromatography (TLC) was performed on alumina plates pre-coated with silica gel (Merck silica gel, 60 F254), which were visualised by the quenching of UV fluorescence when applicable (λ_{max} = 254 nm and/or 366 nm) and/or by spraying with vanillin in acidic ethanol followed by heating with a heat gun. NMR spectra were recorded on a Bruker Avance (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR) spectrometer system. Data were analysed via TopSpin 3.6.1 software package. Spectra were referenced to TMS or residual solvent ($CDCl_3$ = 7.26 ppm in ^{1}H spectroscopy, and 77.0 ppm in ^{13}C spectroscopy; MeOD-D_4 = 4.78, 3.31 ppm in ^{1}H spectroscopy, and 49.2 ppm in ^{13}C spectroscopy). Fourier transform infrared (FT-IR) spectra were recorded by Perkin Elmer FT-IR spectroscopy (Perkin Elmer, Waltham, MA, USA) in the frequency range of 4000 – 400 cm^{-1} using the ATR method.

Chemistry

(4-aminophenyl)(phenyl)methanol (**2**): A 4-aminobenzophenone (**1**) (4.11 g, 20.86 mmol) was treated with $NaBH_4$ (4 equiv.) in the mixture of THF (100 mL) and H_2O (30 mL). While the mixture was heated under reflux overnight, the colour of the mixture gradually changed from pale yellow to colourless. The reaction mixture was quenched with saturated aqueous ammonium chloride (NH_4Cl) (20 mL). The layers were then separated, and the aqueous layer was extracted with ethyl acetate (EtOAc) (3×20 mL). The combined organic solvents were then dried over sodium sulphate (Na_2SO_4), filtered, and collected. The product was directly used without further purification (Supporting information, S1-S2). Brownish solid. Yield: (3.96 g, 95%). **IR ($\tilde{\nu}$/cm^{-1}):** 3406 (NH stretching), 3055 (Csp^2-H stretching), 1613, 1513, 1277 (C-O stretching), 1174, 746. **^{1}H NMR (500 MHz, CD_3OD, δ/ppm):** 5.67 (s, 1H, H-7), 6.70 (d, *J*= 8.4 Hz, 2H, H-3, H-5), 7.09 (d, *J*= 8.4 Hz, 2H, H-2, H-6), 7.22 (t, *J*= 7.5 Hz, 1H, H-11), 7.30 (t, *J*= 7.5 Hz, 2H, H-10, H-12), 7.37 (d, *J*= 7.5 Hz, 2H, H-9, H13). **^{13}C NMR (125 MHz, CD_3OD, δ/ppm):** 77.0, 116.5, 127.7, 128.0, 129.0, 129.2, 135.7, 146.5, 148.0.

Synthesis of 4-amidobenzhydryl analogues 3a-3f

A 4-aminobenzhydrol **2** (1.0 equiv.) was dissolved in 20 mL of dry dichloromethane (DCM) and stirred in an ice bath. The 4-dimethylaminopyridine (DMAP) (2.2 equiv.), triethylamine (Et_3N) (2.2 equiv.) and acetic anhydride/acyl chloride (2.2 equiv.) were added dropwise while stirring the reaction solution. The ice bath was removed, and the mixture was allowed to stir at room temperature overnight. The reaction progress was monitored by TLC (1:4, *n*-hexane: ethyl acetate). After stirring at room temperature overnight, the reaction mixture was quenched with saturated aqueous ammonium chloride (NH_4Cl) (20 mL). The layers were then separated, and the aqueous layer was extracted with ethyl acetate (EtOAc) (3×20 mL). The combined organic solvents were then dried over sodium sulphate (Na_2SO_4), filtered, and collected. The excess volatile solvent was removed under reduced pressure by using a rotary evaporator then gave the product **3** after column chromatography (Supporting information, S3-S14).

(4-acetamidophenyl)(phenyl)methyl acetate (**3a**): A 4-aminobenzhydrol **2** (0.219 g, 1.10 mmol) was

dissolved in 10 mL of dry dichloromethane (DCM) and stirred in an ice bath. The 4-dimethylaminopyridine (DMAP) (0.268 g, 2.20 mmol), triethylamine (Et_3N) (0.31 mL, 2.22 mmol) and acetic anhydride (0.21 mL, 2.20 mmol) were added dropwise while stirring the reaction solution. The reaction then proceeded and worked up according to the general procedure. White solid. Yield: (0.23 g, 74%). **IR ($\tilde{\nu}$/cm^{-1}):** 3242 (NH stretching), 3049 (Csp^2-H stretching), 1725 (C=O stretching), 1662 (NHC=O amide), 1513, 1369, 1225 (C-O stretching), 752. **^{1}H NMR (500 MHz, $CDCl_3$, δ/ppm):** 2.09 (s, 6H, H-2", H-2'), 6.78 (s, 1H, H-7), 7.22 (d, *J*= 8.6 Hz, 2H, H-3, H-6), 7.23-7.28 (m, 5H, H-9–H-13), 7.40 (d, *J*= 8.6 Hz, 2H, H-3, H-5). **^{13}C NMR (125 MHz, $CDCl_3$, δ/ppm):** 21.3, 24.5, 76.6, 120.0, 127.0, 127.8, 127.9, 128.5, 136.0, 137.6, 140.0, 168.6, 170.2.

(4-benzamidophenyl)(phenyl)methyl benzoate(**3b**): A 4-aminobenzhydrol **2** (0.210 g, 1.06 mmol) was dissolved in 10 mL of dry dichloromethane (DCM) and stirred in an ice bath. The 4-dimethylaminopyridine (DMAP) (0.268 g, 2.20 mmol), triethylamine (Et_3N) (0.31 mL, 2.22 mmol) and benzyl chloride (0.26 mL, 2.20 mmol) were added dropwise while stirring the reaction solution. The reaction then proceeded and worked up according to the general procedure. Brownish solid. Yield: (0.28 g, 63%). **IR ($\tilde{\nu}$/cm^{-1}):** 3374 (NH stretching), 3029 (Csp^2-H stretching), 1717 (C=O stretching), 1654 (NHC=O amide), 1510, 1274 (C-O stretching), 1102, 760. **^{1}H NMR (500 MHz, $CDCl_3$, δ/ppm):** 7.04 (s, 1H, H-7), 7.23 (t, *J*= 7.4 Hz, 1H, H-11), 7.29 (t, *J*= 7.4 Hz, 2H, H-10, H-12), 7.36-7.53 (m, 10H, H-2, H-6, H-9, H-13, H-4'-H-6', H-4″-H-6″), 7.57 (d, *J*= 8.7 Hz, 2H, H-3, H-5), 7.75 (s, NH), 7.79 (d, *J*= 8.5 Hz, 2H, H-3', H-7'), 8.08 (d, *J*= 8.5 Hz, 2H, H-3″, H-7″). **^{13}C NMR (125 MHz, $CDCl_3$, δ/ppm):** 77.1, 120.1, 127.0, 127.1, 128.0, 128.1, 128.4, 128.6, 128.9, 129.8, 130.2, 132.0, 133.2, 134.9, 136.5, 137.6, 140.1, 165.6, 165.8.

(4-butyramidophenyl)(phenyl)methyl butyrate (**3c**): A 4-aminobenzhydrol **2** (0.209 g, 1.05 mmol) was dissolved in 10 mL of dry dichloromethane (DCM) and stirred in an ice bath. The 4-dimethylaminopyridine (DMAP) (0.268 g, 2.20 mmol), triethylamine (Et_3N) (0.31 mL, 2.22 mmol) and butyryl chloride (0.25 mL, 2.20 mmol) were added dropwise while stirring the reaction solution. The reaction then proceeded and worked up according to the general procedure. Yellowish oil. Yield: (0.32 g, 81%). **IR ($\tilde{\nu}$/cm^{-1}):** 3308 (NH stretching), 2964 (Csp^2-H stretching), 1737 (C=O stretching), 1668 (NHC=O amide), 1504, 1252 (C-O stretching), 1171, 746. **^{1}H NMR (500 MHz, $CDCl_3$, δ/ppm):** 0.85 (t, *J*= 7.4 Hz, 3H, H-4″), 0.88 (t, *J*= 7.4 Hz, 3H, H-4'), 1.60 (m, 2H, H-3″), 1.63 (m, 2H, H-3'), 2.21 (t, *J*= 7.4 Hz, 2H, H-2"), 2.31 (t, *J*= 7.4 Hz, 2H, H-2'), 6.76 (s, 1H, H-7), 7.19 (d, *J*= 8.5 Hz, 2H, H-2, H-6), 7.17-7.24 (m, 5H, H-9–H-13), 7.41 (d, *J*= 8.5 Hz, 2H, H-3, H-5), 7.56 (s, NH). **^{13}C NMR (125 MHz, $CDCl_3$, δ/ppm):** 13.6, 13.7, 18.5, 19.1, 36.5, 39.6, 76.3, 119.8, 126.9, 127.9, 128.5, 136.1, 137.7, 140.3, 171.5, 172.8.

(4-isobutyramidophenyl)(phenyl)methyl isobutyrate (**3d**): A 4-aminobenzhydrol **2** (0.220 g, 1.10 mmol) was dissolved in 20 mL of dry dichloromethane (DCM) and stirred in an ice bath. The 4-dimethylaminopyridine (DMAP) (0.268 g, 2.20 mmol), triethylamine (Et_3N) (0.31 mL, 2.22 mmol) and isobutyryl chloride (0.23 mL, 2.20 mmol) were added dropwise while stirring the reaction solution. The reaction then proceeded and worked up according to the general procedure. Brownish solid. Yield: (0.36 g, 90%). **IR ($\tilde{\nu}$/cm^{-1}):** 3291 (NH stretching), 2970 (Csp^2-H stretching), 1726 (C=O stretching), 1654 (NHC=O amide), 1533, 1461, 1192 (C-O stretching), 1117, 746, 694. **^{1}H NMR (500 MHz, $CDCl_3$, δ/ppm):** 1.19 (dd, *J*= 1.8, 7.0 Hz, 6H, H-3′, H-4′), 1.24 (d, *J*= 7.0 Hz, 6H, H-3″, H-4″), 2.42 (m, 1H, H-2″), 2.59 (m, 1H, H-2′), 6.75 (s, 1H, H-7), 7.12 (br s, 1H, NH), 7.29 (d, *J*= 8.5 Hz, 2H, H-2, H-6), 7.30-7.34 (m, 5H, H-9–H-13), 7.50 (d, *J*= 8.5 Hz, 2H, H-3, H-5). **^{13}C NMR (125 MHz, $CDCl_3$, δ/ppm):** 18.9, 19.7, 34.2, 36.7, 76.1, 119.7, 126.9, 127.8, 127.9, 128.5, 136.2, 137.6, 140.3, 175.2, 176.0.

(4-pentanamidophenyl)(phenyl)methyl pentanoate (**3e**): A 4-aminobenzhydrol **2** (0.220 g, 1.10 mmol) was dissolved in 20 mL of dry dichloromethane (DCM) and stirred in an ice bath. The 4-dimethylaminopyridine (DMAP) (0.268 g, 2.20 mmol), triethylamine (Et_3N) (0.31 mL, 2.22 mmol) and valeroyl chloride (0.24 mL, 2.20 mmol) were added dropwise while stirring the reaction solution. The reaction then proceeded and worked up according to the general procedure. Yellowish oil. Yield: (0.34 g, 85%). **IR ($\tilde{\nu}$/cm^{-1}):** 3308 (NH stretching), 3032 (Csp^2-H stretching), 2932 (Csp^3-H stretching), 1737 (C=O stretching), 1665 (NHC=O amide), 1522, 1413, 1249 (C-O stretching), 1168, 743, 697. **^{1}H NMR (500 MHz, $CDCl_3$, δ/ppm):** 0.82 (t, *J*= 7.4 Hz, 3H, H-5″), 0.86 (t, *J*= 7.4 Hz, 3H, H-5'), 1.25 (m, 2H, H-4″), 1.31 (m, 2H, H-4'), 1.56 (m, 2H, H-3″), 1.62 (m, 2H, H-3'), 2.26 (t, *J*= 7.7 Hz, 2H, H-2″), 2,34 (t, *J*= 7.6 Hz, 2H, H-2'), 6.77 (s, 1H, H-7), 7.20 (d, *J*= 8.5 Hz, 1H, H-2, H-6), 7.22-7.27 (m, 5H, H-9–H-13), 7.41 (d, *J*= 8.5 Hz, 1H, H-3, H-5). **^{13}C NMR (125 MHz, $CDCl_3$, δ/ppm):** 13.7, 13.8, 22.2, 22.4, 27.0, 27.7, 34.3, 37.5, 76.3, 119.8, 127.0,

127.8, 127.9, 128.5, 136.1, 137.6, 140.2, 171.6, 172.9

(4-(cyclohexanecarboxamido)phenyl)(phenyl)methyl cyclohexanecarboxylate (**3f**): A 4-aminobenzhydrol **2** (0.220 g, 1.10 mmol) was dissolved in 20 mL of dry dichloromethane (DCM) and stirred in an ice bath. The 4-dimethylaminopyridine (DMAP) (0.268 g, 2.20 mmol), triethylamine (Et_3N) (0.31 mL, 2.22 mmol) and cyclohexanecarbonyl chloride (0.26 mL, 2.20 mmol) were added dropwise while stirring the reaction solution. The reaction then proceeded and worked up according to the general procedure. Brownish solid. Yield: (0.449 g, 91%). **IR (ṽ/cm^{-1}):** 3259 (NH stretching), 3029 (Csp^2-H stretching), 2926 (Csp^3-H stretching), 2851, 1737 (C=O stretching), 1662 (NHC=O amide), 1539, 1507, 1249 (C-O stretching), 1160, 726, 694. **^{1}H NMR (500 MHz, $CDCl_3$, δ/ppm):** 1.20-1.34 (m, 6H, H-4a', H-6a', H-4a'', H-6a'', H-5a', H-5a''), 1.46 (m, 2H, H-3a'', H-7a''), 1.53 (m, 2H, H-3a', H-7a'), 1.68-1.77 (m, 4H, 5b', H-5b'', H-4b'', H-6b''), 1.81-1.85 (m, 2H, H-4b', H-6b'), 1.91-1.97 (m, 2H, H-3b', H-7b', H-3b'', H-7b''), 2.21 (tt, *J*= 3.4, 11.8 Hz, 1H, H-2''), 2.40 (tt, *J*= 3.4, 11.8 Hz,, 1H, H-2'), 6.82 (s, 1H, H-7), 7.16 (br s, 1H, NH), 7.26 (d, *J*= 8.5 Hz, 1H, H-2, H-6), 7.29-7.33 (m, 5H, H-9–H-13), 7.49 (d, *J*= 8.5 Hz, 1H, H-3, H-5). **^{13}C NMR (125 MHz, $CDCl_3$, δ/ppm):** 25.4, 25.6, 25.7, 29.0, 29.6, 43.3, 46.6, 76.0, 119.7, 126.9, 127.7, 127.8, 128.5, 136.2,137.6, 140.4, 174.4, 175.0.

Biological assay

Cell culture

Kasumi-1 cells were purchased from the American Type Culture Collection (ATCC Manassa, VA, USA). Kasumi-1 cells were maintained in RPMI 1640 medium containing L-Glutamine supplemented with 10% of fetal bovine serum and 1% of Penicillin-Streptomycin. The cells were subcultured at least twice a week (48-72hrs) or when 80% confluent with a dilution of 1:10 and kept in T-25 or T-75 flasks.

Cytotoxic evaluation of 4-amidobenzhydryl analogues

The effects of 4-amidobenzhydryl analogues (3a-3f) on Kasumi-1 cell viability were examined using the thiazolyl blue tetrazolium bromide (MTT) assay [16]. The cells were then seeded in clear round bottom 96-well microplates at a seeding density of 1×10^4 cells per well in 100 µL of cell suspension per well. The cells were treated with serial dilutions of the synthesised compounds in DMSO at final concentrations of 200 µg/mL, 100 µg/mL, and 50 µg/mL. Cells treated with 0.1% DMSO served as the vehicle control. The treatments were carried out for durations of 24, 48, and 72 hours. After exposure times, the plate was centrifuged, and the media was aspirated and replaced with fresh media with 10% of 5 mg/mL MTT solution. The microplate was then incubated at 37°C for 4 hours. Then, the media was once again aspirated. 70 µL of DMSO was then added into each well and the absorbance was measured at 570 nm with a reference wavelength of 620 nm. The IC_{50} value of each treatment group was obtained by referencing the treatment concentration needed to reduce the cell viability to 50% using the dose-response curve. All treatments were performed in triplicate and repeated in three independent experiments

The cell viability was calculated using the following formula:

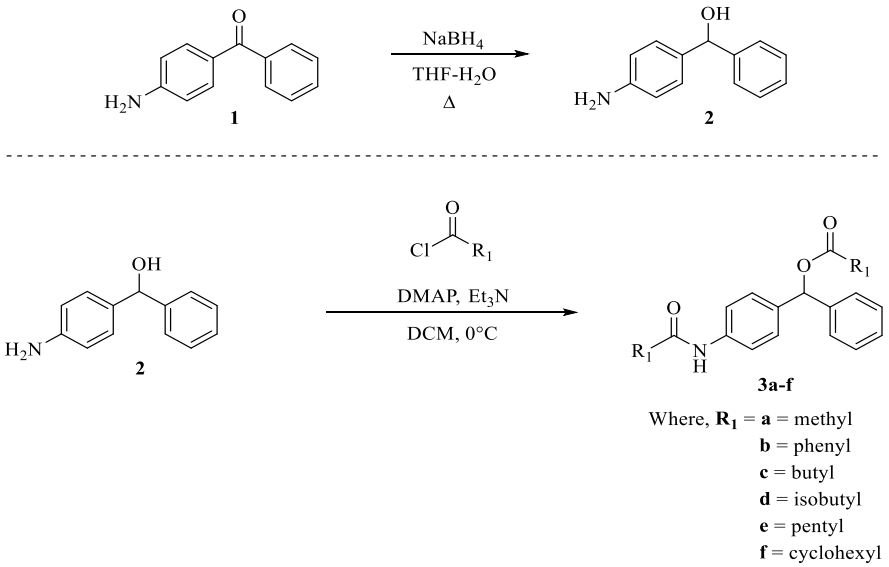
$$Cell\ Viability\ (\%) = \frac{Treated\ Cells\ Absorbance^{570-620}}{Vehicle\ Control\ Absorbance^{570-620}} \times 100 \quad (1)$$

Results and Discussion

Synthesis of 4-amidobenzhydryl analogues

Scheme 1 shows the synthetic pathway for the preparation of 4-amidobenzhydryl analogues (**3a–3f**). The synthesis began with the preparation of (4-aminophenyl)(phenyl)methanol (**2**), which was achieved by reducing (4-aminophenyl)(phenyl) methanone (**1**) with sodium borohydride ($NaBH_4$) in a THF–H_2O solvent system under heating. In the next step, 4-amidobenzhydryl analogues (**3a–3f**) were synthesised through an acylation reaction. This was accomplished by reacting (4-aminophenyl)(phenyl) methanol (**2**) with various acyl chlorides in the presence of dry dichloromethane (DCM) as the solvent. The reaction was catalysed by 4-dimethylaminopyridine (DMAP) and facilitated by triethylamine (Et_3N) as a base at 0 °C. The chemical structures of the synthesised target compounds were confirmed through comprehensive spectroscopic analyses, including FT-IR and NMR techniques.

From the characterisation analysis conducted, the structural confirmation of the target compounds is successfully validated. This can be observed through the presence of the C=O carbon peak in the ^{13}C-NMR spectra in range of 146.0 to 180.0 ppm. From the FT-IR spectra, the presence of a sharp C=O carbonyl and NHC=O amide peaks at range 1600 to 1740 cm^{-1} can also be observed, further confirming the structure of the target compounds. For example, the ^{13}C-NMR spectrum for compound **3a** visualises the peak for C=O carbons at 168.6 and 170.2 ppm, while from the FT-IR spectrum, peaks observed at 1725 and 1662 cm^{-1} were assigned to functional groups C=O and NHC=O respectively.



Scheme 1. Synthesis strategy for the preparation of 4-amidobenzyhydryl analogues **3a-f**

Cytotoxic evaluation

The synthesised compounds demonstrated significant cytotoxic effects against Kasumi-1 cells, as assessed using the MTT assay (**Table 1** and **Figure 1**). After 72 hours of exposure, all tested compounds reduced cell viability notably. Among them, **3a** is particularly effective, showing notable reductions in cell viability even after 24 hours of treatment (**Figure 1**). **3a** consistently exhibited cytotoxic activity across all time points, with IC_{50} values of 247.35 µM at 24 hours, 106.01 µM at 48 hours, and 98.94 µM at 72 hours. Other compounds, such as **3e** displayed time-dependent cytotoxicity, with their effects becoming prominent only at 48 and 72 hours. The IC_{50} values for **3e** were 272.48 µM and 98.09 µM at 48 and 72 hours, respectively. The delayed activity of these compounds suggests a unique mechanism of action that justifies further investigation. These results highlight **3a** as the most promising compound due to its consistent efficacy across all time points. Its superior performance, coupled with stable activity, positions **3a** as a lead candidate for further development. The time-dependent cytotoxic effects observed with **3e** suggest they may offer targeted applications, potentially through mechanisms that take effect over prolonged exposure durations. However, it is important to note that the IC_{50} values are derived from triplicate experiments and represent descriptive comparisons only.

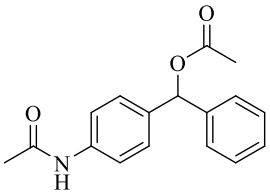
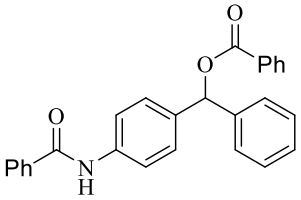
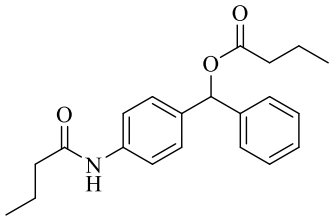
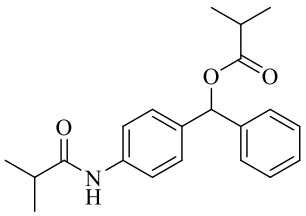
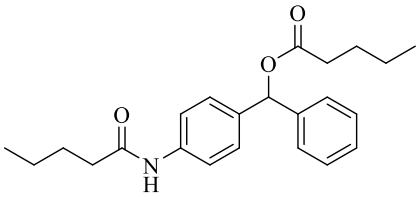
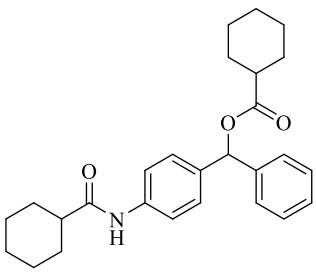
While these findings provide valuable insights into the anticancer potential of the analogues, it is essential to note that this is a preliminary screening conducted on a single cell line, Kasumi-1. Further studies involving additional cancer cell lines and normal cell lines are necessary to validate the selectivity and broad-spectrum efficacy of these compounds. Additionally, future studies should incorporate a known chemotherapeutic agent as a positive control to enable direct comparison of cytotoxic potency. Mechanistic studies, such as apoptosis assays or pathway analyses, will also be essential to elucidate the mode of cell death induced by these analogues.

Structural activity relationship study (SAR study)

All compounds exhibited cytotoxic activity, with IC_{50} values ranging from 82.6 µM to 98.9 µM after 72 hours of treatment. The methyl-substituted derivative **3a** showed significant cytotoxic effects against Kasumi-1 cells, with IC_{50} values of 247.3 µM at 24 hours, 106.0 µM at 48 hours, and 98.9 µM at 72 hours. To investigate the impact of substitutions, different functional groups were introduced at the carboxamide and ester positions of the 4-amidobenzhydryl analogues.

Initially, the cytotoxic effect of the methyl group was evaluated. The introduction of a methyl group (**3a**) at the carboxamide and ester positions enhanced cytotoxic activity compared to other substituted derivatives. However, the cytotoxic activity observed methyl-substituted derivative **3a** and the butyl (**3c**) and isobutyl (**3d**) derivatives appeared comparable within the tested concentration range. In contrast, phenyl substituted derivatives **3b** and cyclohexyl derivatives **3f** showed no cytotoxic activity at 24 and 48 hours. Substitution with butyl (**3c**) and isobutyl (**3d**) groups reduced potency, indicating that these groups were less favourable.

Table 1. IC_{50} value of 4-amidobenzyhydryl analogues **3a-3f**

	IC_{50} Value (µM)		
	24 hrs	**48 hrs**	**72 hrs**
 **3a**	247.35	106.01	98.94
 **3b**	NA	NA	86.00
 **3c**	NA	147.58	89.06
 **3d**	412.98	147.49	82.60
 **3e**	NA	272.48	98.09
 **3f**	NA	NA	88.31

NA signifies undetermined IC_{50} value. All experiments were conducted in triplicates

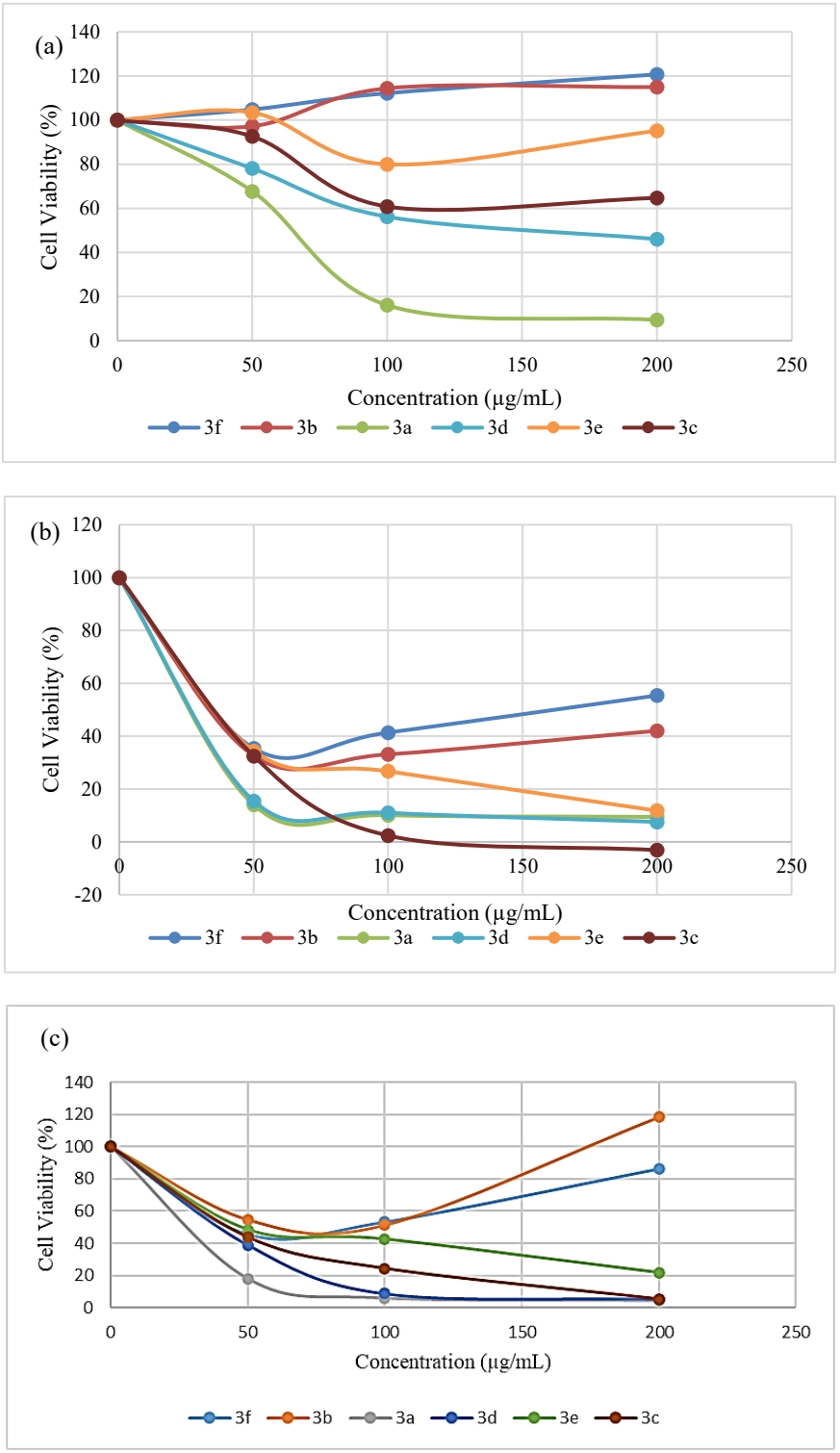


Figure 1. Dose-response curves of Kasumi-1 cells when treated with 4-amidobenzyhydryl analogues. Cells were exposed to various concentrations of each compound for (A) 24 hrs, (B) 48 hrs, and (C) 72 hrs. Cytotoxicity was assessed using the MTT assay. Each data point represents the mean cell viability (%) from three independent experiments

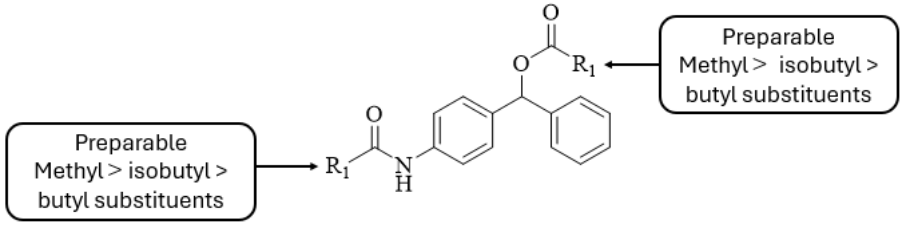


Figure 2. Structure activity relationship of 4-amidobenzhydryl analogues

Furthermore, compounds **3c** (IC_{50} = 147.58 µM at 48 hours; 89.06 µM at 72 hours) and **3e** (IC_{50} = 272.48 µM at 48 hours; 82.6 µM at 72 hours) exhibited cytotoxic activity, although less effectively than **3a**. These findings suggest that aliphatic substitutions are generally more favourable for cytotoxic activity. Interestingly, cytotoxicity was observed to decrease as the length of the carbon chain in the substituent increased. Additionally, the introduction of bulky groups such as phenyl or cyclohexyl rings reduced cytotoxic potency, likely due to steric hindrance interfering with the interaction of the compounds with their biological targets. It may also be caused by the conformational flexibility aliphatic chains offer compared to aromatic or cyclic substituents, possibly allowing for more favourable conformation for optimal binding with the biological targets (**Figure 2**).

Conclusion

In summary, the synthesised 4-amidobenzhydryl analogues demonstrated significant anticancer potential against human myeloid leukemia Kasumi-1 cells, with promising IC_{50} values observed after 72 hours of treatment. Cytotoxicity evaluations revealed that the efficacy of compound **3a** in reducing cell viability was most consistent across all time points compared to the other analogues, distinguishing the compound as the most potent analogue. The structure-activity relationship study highlighted that aliphatic substitutions at the carboxamide and ester positions enhanced cytotoxic activity, in contrast to substitutions such as phenyl and cyclohexyl groups, which reduced the cytotoxicity. This supports the cytotoxic evaluation of the analogue compounds. These findings suggest that 4-amidobenzhydryl analogues, especially compound 3a, could serve as lead candidates for further development as anticancer agents.

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