Design, synthesis and cytotoxicity of novel N-benzylpiperidin-4-one oximes on human cervical cancer cells

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Abstract. A series of fifteen diversified N-benzylpiperidin-4-one oximes were synthesized and characterized by their NMR spectral data. Additionally, single-crystal XRD analysis was performed for the representative symmetrically and unsymmetrically substituted molecules. All the synthesized oximes from unsymmetrical ketones existed as E-isomer as witnessed by their NMR and XRD data. Among the synthesized target compounds that evaluated for their *in vitro* cytotoxicity against human cervical carcinoma (HeLa) cells, five compounds were potent with IC₅₀ < 17 μ M. 1-Benzyl-2,6-bis(4-isopropylphenyl)-3-methylpiperidin-4-one oxime **3c** with an IC₅₀ of 13.88 μ M was found to be the best active compound as depicted by the microscopic analysis.

Keywords. N-benzylpiperidin-4-one oximes; cytotoxicity, HeLa cells; single-crystal XRD.

1. Introduction

Cervical cancer, a slow growing squamous cell carcinoma, caused by human papillomavirus (HPV) is the second most common cancer after breast cancer that affects women.^{1–3} Although a number of new and effective medicines have been implemented in the cancer arena, some limitations exist in present therapeutic regime such as target non-specificity, associated side effects and lack of potency.^{4,5} Hence the need of new, safe and potent anticancer compounds is motivating the medicinal chemists towards the invention and development of effective molecules. Considering the reports on piperidin-4-one heterocycles (figure 1a and b) as good cytotoxic agents,^{6–10} we synthesized some N-benzylated piperidin-4-one oximes (figure 1c) as target molecules.

In fact, the chalcones with piperidone core (**a**) are the heterocyclic analogs of α,β -unsaturated ketones (curcumin). They are reported to possess a strong cytotoxic

and anticancer property over a wide range of cancer cells.^{11–13} The structure–activity relationship (SAR) studies from differently substituted piperidones reveal that the nature of functional groups and position of the substituents are important to effect the biological actions.^{6–10}

2. Experimental

2.1 NMR experiments

All 1D NMR spectra of the synthesized compounds were recorded on Jeol JNM ECP 400 NMR spectrometer at 294 K. The ¹H and ¹³C NMR spectra were measured, respectively 0.03 M and 0.05 M solutions in CDCl₃ with TMS as internal reference in 5 mm NMR tubes. The pulse conditions were as follows: ¹H NMR spectra: SF 399.78 MHz, AQ 2.73 s, NS 32, DS 0, SW 5998.8 Hz, pulse 4.65 μ s, angle 45°, width 9.3 μ s, DR 0.366 Hz, RD 5 s, RG 13, data points 16384, pre scan delay 1 s; ¹³C NMR spectra: SF 100.52 MHz, AQ 1.25 s, NS 250, DS 4, SW 26178.01 Hz, Pulse 3.13 μ s,

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Figure 1. General structure of literature compounds (a) and (b) with good cytotoxicity against various cell line and synthesized target compounds (c).

angle 30°, width 9.4 μ s, DR 0.798 Hz, RD 1 s, RG 25, data points 32768, pre scan delay 1 s. The ^1H and ^{13}C chemical shift values are given in δ scale (ppm) and referred to TMS, via the solvent signals (^1H , residual CHCl_3 at 7.26 ppm; ^{13}C , CHCl_3 at 77.16 ppm). Coupling constants J are reported in Hz.

2.2 Single-crystal XRD

The X-ray diffraction quality crystals of **3d** and **3n** were obtained by slow evaporation from chloroform and the crystal structure was determined by the X-ray diffraction data collected on a Bruker SMART APEX CCD diffractometer.¹⁴ X-ray analysis (**3d** and **3n**) were performed at 296 K temperature using Mo-K α radiation ($\lambda = 0.7107 \text{ \AA}$) to a maximum θ range of 25°. Crystal to detector distance 6.05 cm, 512 $\times$ 512 pixels/frame, oscillation/frame -0.3° , maximum detector swing angle = -30.0° , beam centre = (260.2, 252.5), in plane spot width = 1.24, SAINT integration. SHELX-97 (ShelxTL) was used for structure solution and full matrix least squares refinement on F^2 .¹⁵ Hydrogen atoms were included in the refinement as per the riding model.

2.3 General procedure for the synthesis of 2,6-diarylpiperidin-4-ones (**1a–1o**)

All the parent 2,6-diarylpiperidin-4-ones were synthesized by adopting the literature precedent of Noller and Baliah¹⁶ through the condensation of respective ketones, aldehydes and ammonium acetate in 1:2:1 ratio. All the synthesized piperidin-4-ones are in good agreement with their NMR data reported earlier.^{17–19}

2.4 General procedure for the synthesis of 1-benzyl-2,6-diarylpiperidin-4-ones (**2a–2o**)

A mixture of respective 2,6-diarylpiperidin-4-ones (0.01 mol), anhydrous potassium carbonate (0.02 mol, 2.76 g) and benzyl bromide (0.015 mol, 1.78 mL) in DMF (20 mL) was stirred at room temperature for 12–36 h. Progress and completion of the reactions were monitored by the TLC. After the completion, an excess of ice-cold water was added and extracted with dichloromethane ($3 \times 15 \text{ mL}$). The organic layer, thus separated was thrice washed with brine solution ($3 \times 10 \text{ mL}$) and dried over anhydrous Na_2SO_4 . Then the organic layer was concentrated in rota to obtain the crude product, followed by purification on silica-gel (Merck 230–400 mesh), using *n*-hexane and ethyl acetate mixture, afforded the pure 1-benzyl-3-alkyl-2,6-diarylpiperidin-4-ones **2a–2o** in good yields 87–92%. All the N-benzylated piperidine-4-ones were characterized by their analytical and spectral data, and confirmed with previous reports.^{18,19}

2.5 General procedure for the synthesis of 1-benzyl-2,6-diarylpiperidin-4-one oximes (**3a–3o**)

A mixture of 1-benzyl-2,6-diarylpiperidin-4-one (0.002 mol), hydroxylamine hydrochloride (0.003 mol) and sodium acetate trihydrate (0.003 mol) were dissolved in ethanol (5 mL). The resulting reaction mixture was refluxed and the formation of the desired compound monitored by TLC analysis. After completion of reaction, solvent was evaporated using rota-vapour, water was added and extracted with ethyl acetate ($3 \times 15 \text{ mL}$). The organic layer was dried (anhydrous Na_2SO_4) and the solvent were evaporated on a rota-vapour. In a few cases, further purification was required; hence, they either recrystallized using ethanol or purified by column chromatography.

2.6 Cytotoxicity assay (MTT assay)

The HeLa cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from BioWhittaker®, whereas fetal bovine serum (FBS) and other cell culture materials were purchased from Gibco BRL Life Technologies, USA. Paraformaldehyde and bisbenzimidazole Hoechst 33342 stain were procured from Sigma–Aldrich Corp., St. Louis, MO, USA, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Biosesang Inc., Korea.

Mononuclear cells from peripheral blood of healthy adults was used to isolated PBMCs using Ficoll-Hypaque (Pharmacia, Freiburg, FRG) density gradient centrifugation.²⁰ Cells were cultured in T-75 tissue culture flasks (Nunc, Denmark) at 37°C in a 5% CO₂ humidified incubator using appropriate media supplemented with DMEM containing 10% heat-inactivated FBS, 100 units/mL Penicillin and 100 µg/mL Streptomycin. Cells were seeded in a 96 well microtiter plate containing 100 µL medium at a final density of 2×10^4 cells/well at identical conditions. After overnight incubation, the cells were treated with different concentrations of test compounds (6.25–100 µg/mL) or DMSO (carrier solvent) in a final volume of 200 µL. After 24 h, 10 µL of MTT (5 mg/mL) was added to each well and the plate was incubated at 37°C in the dark for 4 h. Then the media along with MTT was removed and the formazan crystals were solubilised by adding DMSO (100 µL/well). Finally, the reduction of MTT was quantified by reading the absorbance at 570 nm by GENios® microplate reader (Tecan Austria GmbH). Effects of the test compounds on cell viability were calculated using cells treated with DMSO as control. The data were subjected to linear regression analysis and the regression lines were plotted for the best straight-line fit.

2.7 Altered morphology study

The altered morphology of exposed cells (1×10^5 cells/well) at IC₅₀ concentration was studied after 24 h using phase contrast microscope (DMI6000B, Leica Microsystems, Wetzlar, Germany). Subsequently, the cells were Hoechst stained to observe the nuclear/chromosomal condensation that occurred after treatment with the test compound. For staining the cells, 96 well cell culture plates were used to culture the cells (1×10^4 cells/well) in three replicates to treat with the ideal lead compound **3c**. Then the cells were incubated at 37°C overnight and the media was removed to wash the cells twice with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for one day at –4°C.¹⁰ Further, the cells were stained with nuclear binding dye Hoechst 33342 (1 µg/mL of the fluorescent DNA-binding dye, bis-benzimide Hoechst 33342 stain was added to the fixed cells and incubated for 20 min at room temperature) to expose the nuclear condensation/aggregation due to the effect of the compound **3c**. Similarly, PI stock solution (1 mg/mL) was diluted to 1:3000 with 1X PBS and added to the cells for allowing the stain to get permeated by the dead cells. The Hoechst and PI stained cells were visualized and photographed under

fluorescence microscope (CTR 6000; Leica, Wetzlar, Germany).

2.8 Annexin V-PI dual staining to access apoptosis

Annexin V-FITC/PI dual staining was performed to access the level of apoptosis and cell death in HeLa cells treated with IC₅₀ concentration 13.88 µM of compound **3c**, 24 h post treatment. Annexin V-FITC and PI staining solutions used were components of the FITC annexin V Apoptosis Detection Kit (BD Pharmingen™). The staining procedure was followed as per manufacturer's instructions. Briefly, HeLa cells incubated at 37°C with 5% CO₂, 0, 4, 12 and 24 h post treatment with 13.88 µM of compound **3c**, were harvested using 1X Trypsin-EDTA solution prepared in serum free DMEM. Cells were washed twice with cold PBS and then resuspended in 1X binding buffer (provided with the kit) at a concentration of 1×10^6 cells/mL. 100 µl of this solution (1×10^5 cells) was transferred to a 5 ml culture tube followed by addition of 5 µl of FITC Annexin V, gentle vortexing and incubation for 15 min at room temperature (25°C) in dark. 2 µl of PI staining solution was then added to these Annexin V-FITC labelled cells and incubated further for 5 min under similar conditions. Finally, 400 µl of 1X binding buffer was added to each tube. Cells were mounted on glass slides and observed under fluorescence microscope (CTR 6000; Leica, Wetzlar, Germany).

2.9 ¹H and ¹³C NMR data of the target compounds

2.9a 1-Benzyl-3-methyl-2,6-diphenylpiperidin-4-one oxime (3a, C₂₅H₂₆N₂O): Faint pink solid; Yield: 89%; m.p.: 176–178°C; ¹H NMR (400 MHz, CDCl₃): δ = 8.59 (s, OH), 7.50 (t, *J* = 9.7 Hz, 4H), 7.37–7.33 (m, 4H), 7.32–7.24 (m, 2H), 7.10 (t, *J* = 3.1 Hz, 3H), 6.83–6.77 (m, 2H), 3.73 (dd, *J* = 3.5, 11.5 Hz, H-6a), 3.57 (d, *J* = 15.0 Hz, 1H of N-CH₂), 3.48–3.36 (m, 3H of H-2a, H-3a and 1H of N-CH₂ merged), 2.58–2.51 (m, H-5a), 2.14 (dd, *J* = 11.7, 13.9 Hz, H-5e), 0.76 (d, *J* = 6.6 Hz, CH₃ at C-3) ppm; ¹³C NMR (100.52 MHz, CDCl₃): δ = 159.64 (C-4), 144.13 (C-6'), 142.54 (C-2'), 136.70 (N-Bn *ipso* carbon), 130.08, 129.10, 128.73, 128.46, 127.84, 127.64, 127.41, 126.67, 72.36 (C-2), 63.96 (C-6), 53.43 (N-CH₂-Ph), 43.90 (C-3), 35.06 (C-5), 12.84 (CH₃ at C-3) ppm; and HRMS: *m/z* calculated: 370.2045, found: 370.4231.

2.9b 1-Benzyl-3-methyl-2,6-bis(4-methylphenyl)piperidin-4-one oxime (3b, C₂₇H₃₀N₂O): Off-white solid;

Yield: 78%; m.p.: 150–152°C; ^1H NMR (400 MHz, CDCl_3): δ = 9.45 (s, OH), 7.40–7.35 (m, 4H), 7.15–7.09 (m, 7H), 6.83–6.82 (m, 2H), 3.67 (dd, J = 3.3, 11.7 Hz, H-6a), 3.56 (d, J = 15.0 Hz, 1H of N-CH₂), 3.47–3.42 (m, 2H of N-CH₂ and H-2a merged), 3.30 (d, J = 9.8 Hz, H-3a), 2.55–2.47 (m, H-5a), 2.34 (s, 3H), 2.33 (s, 3H), 2.16–2.09 (unresolved quartet, H-5e), 0.76 (d, J = 6.6 Hz, CH₃ at C-3) ppm; ^{13}C NMR (100.52 MHz, CDCl_3): δ = 159.81 (C-4), 141.12 (C-6'c), 139.45 (C-2'c), 137.09 (C-6'), 136.86 (C-2'), 136.67 (N-Bn *ipso* carbon), 130.10, 129.35, 129.07, 127.51, 71.92 (C-2), 63.55 (C-6), 53.08 (N-CH₂-Ph), 43.91 (C-3), 35.27 (C-5), 21.28 (CH₃ at C-6d and C-2d) 12.84 (CH₃ at C-3) ppm; and HRMS: m/z calculated: 398.2358, found: 398.2833.

2.9c *1-Benzyl-2,6-bis(4-isopropylphenyl)-3-methylpiperidin-4-one oxime (3c, C₃₁H₃₈N₂O)*: White solid; Yield: 90%; m.p.: 206–208°C; ^1H NMR (400 MHz, CDCl_3): δ = 7.31 (d, J = 8.0 Hz, 4H), 7.15 (d, J = 7.7 Hz, 2H), 7.10 (unresolved dt, 5H), 6.93 (d, J = 6.9 Hz, 2H), 3.92 (d, J = 6.2 Hz, H-6a), 3.79 (d, J = 6.2 Hz, H-2a), 3.69 (d, J = 14.2 Hz, 1H of N-CH₂), 3.59–3.51 (m, 2H of N-CH₂ and H-3a merged), 2.91–2.81 (m, 2H 2CH of *iPr*), 2.62–2.55 (m, 1H of H-5a), 1.23 (t, J = 7.5 Hz, 13H), 1.00 (d, J = 6.9 Hz, CH₃ at C-3) ppm; ^{13}C NMR (100.52 MHz, CDCl_3): δ = 159.96 (C-4), 148.08 (C-6'c), 147.87 (C-2'c), 141.36 (C-6'), 139.71 (C-2'), 136.78 (N-Bn *ipso* carbon), 129.89, 127.48, 126.59, 126.32, 72.73 (C-2), 64.38 (C-6), 53.89 (N-CH₂-Ph), 43.82 (C-3), 35.04 (C-5), 33.90 (CH of *iPr* at C-2d, C-6d), 24.23 (2CH₃ at C-6d), 24.16 (2CH₃ at C-2d), 12.87 (CH₃ at C-3) ppm; and HRMS: m/z calculated: 454.2984, found: 454.3362.

2.9d *1-Benzyl-2,6-bis(4-chlorophenyl)-3-methylpiperidin-4-one oxime (3d, C₂₅H₂₄Cl₂N₂O)*: Off-white solid; Yield: 82%; m.p.: 174–176°C; ^1H NMR (400 MHz, CDCl_3): δ = 7.43–7.39 (m, 4H), 7.30 (d, J = 6.2 Hz, 4H), 7.11 (d, J = 5.5 Hz, 3H), 6.78–6.72 (m, 2H), 3.69 (d, J = 7.3 Hz, H-6a), 3.52–3.32 (m, 4H of H-2a, H-3a and 2H of N-CH₂ merged), 2.53–2.45 (m, H-5a), 2.14–2.06 (m, 1H), 0.75 (d, J = 5.8 Hz, CH₃ at C-3) ppm; ^{13}C NMR (100.52 MHz, CDCl_3): δ = 158.92 (C-4), 142.30 (C-6'c), 140.75 (C-2'c), 136.45 (N-Bn *ipso* carbon), 133.30 (C-6'), 133.04 (C-2'), 130.27, 129.75, 129.10, 128.94, 128.71, 127.80, 126.86, 71.98 (C-2), 63.55 (C-6), 53.85 (N-CH₂-Ph), 43.67 (C-3), 34.72 (C-5), 12.74 (CH₃ at C-3) ppm; and HRMS: m/z calculated: 338.1266, found: 338.1854.

2.9e *1-Benzyl-2,6-bis(4-methoxyphenyl)-3-methylpiperidin-4-one oxime (3e, C₂₇H₃₀N₂O₃)*: Pale yellow solid; Yield: 86%; m.p.: 142–144°C; ^1H NMR (400 MHz, CDCl_3): δ = 7.40–7.35 (m, 4H), 7.08 (t, J = 2.7 Hz, 3H), 6.87–6.81 (m, 6H), 3.80 (s, 3H), 3.79 (s, 3H), 3.66 (dd, J = 3.3, 11.6 Hz, H-6a), 3.54 (d, J = 14.6 Hz, 1H of N-CH₂), 3.47–3.42 (m, 2H of H-2a and 1H of N-CH₂ merged), 3.28 (d, J = 9.8 Hz, H-3a), 2.55–2.48 (m, H-5a), 2.13 (t, J = 12.6 Hz, H-5e) 0.75 (d, J = 6.6 Hz, CH₃ at C-3) ppm; ^{13}C NMR (100.52 MHz, CDCl_3): δ = 159.89 (C-4), 158.94 (C-6'c), 158.80 (C-2'c), 137.29 (C-6'), 136.15 (C-2'), 134.49 (N-Bn *ipso* carbon), 129.97, 129.85, 128.84, 127.53, 126.45, 72.10 (C-2), 63.67 (C-6), 55.38 (CH₃O at C-6'c, C-2'c), 53.45 (N-CH₂-Ph), 43.98 (C-3), 35.19 (C-5), 12.81 (CH₃ at C-3) ppm; and HRMS: m/z calculated: 430.2256, found: 430.3451.

2.9f *1-Benzyl-2,6-bis(4-ethoxyphenyl)-3-methylpiperidin-4-one oxime (3f, C₂₉H₃₄N₂O₃)*: White solid; Yield: 86%; m.p.: 158–160°C; ^1H NMR (400 MHz, CDCl_3): δ = 8.89 (s, OH), 7.35 (d, J = 8.4 Hz, 4H), 7.08 (s, 3H), 6.85 (d, J = 6.6 Hz, 4H), 6.82–6.81 (m, 2H), 4.04–4.01 (m, 4H), 3.64 (d, J = 8.4 Hz, H-6a), 3.54 (d, J = 15.0 Hz, 1H), 3.45–3.41 (m, 2H), 3.27 (d, J = 9.5 Hz, H-3a), 2.50 (t, J = 8.0 Hz, H-5a), 2.16–2.08 (m, H-5e), 1.41 (d, J = 3.2 Hz, 6H), 0.75 (d, J = 5.4 Hz, CH₃ at C-3) ppm; ^{13}C NMR (100.52 MHz, CDCl_3): δ = 159.70 (C-4), 158.18 (C-6'c), 158.80 (C-2'c), 137.64 (C-6'), 135.87 (C-2'), 133.83 (N-Bn *ipso* carbon), 129.92, 128.83, 127.52, 126.44, 114.56, 114.28, 72.04 (C-2), 63.62 (C-6), 54.13 (CH₃CH₂O at C-6'c, C-2'c), 53.37 (N-CH₂-Ph), 44.00 (C-3), 35.15 (C-5), 15.01 (CH₃CH₂O at C-6'c, C-2'c), 12.81 (CH₃ at C-3) ppm.

2.9g *1-Benzyl-2,6-bis(4-benzyloxyphenyl)-3-methylpiperidin-4-one oxime (3g, C₃₉H₃₈N₂O₃)*: White solid; Yield: 93%; m.p.: 175–177°C; ^1H NMR (400 MHz, CDCl_3): δ = 8.30 (s, OH), 7.44–7.33 (m, 14H), 7.08 (d, J = 2.9 Hz, 3H), 6.94 (d, J = 6.2 Hz, 4H), 6.81 (m, 2H), 5.05 (s, 2H), 4.99 (s, 2H), 3.65 (d, J = 10.2 Hz, H-6a), 3.54 (d, J = 15.0 Hz, 1H), 3.45–3.42 (m, 2H), 3.29 (d, J = 9.8 Hz, H-3a), 2.53–2.49 (m, H-5a), 2.15 (t, J = 12.6 Hz, H-5e), 0.76 (d, J = 6.2 Hz, CH₃ at C-3) ppm; ^{13}C NMR (100.52 MHz, CDCl_3): δ = 159.95 (C-4), 158.22 (C-6'c), 158.00 (C-2'c), 137.36 (N-Bn *ipso* carbon), 137.20 (O-Bn *ipso* carbon), 136.51 (C-6'), 134.88 (C-2'), 130.04, 129.89, 128.73, 128.10, 127.71, 127.67, 127.56, 114.97, 114.69, 72.16 (C-2), 70.23 (O-CH₂-Ph at C-6'c, C-2'c), 63.74 (C-6),

54.24 (N- $\underline{\text{CH}_2}$ -Ph), 43.96 (C-3), 35.15 (C-5), 12.85 ($\underline{\text{CH}_3}$ at C-3) ppm.

2.9h *1-Benzyl-2,6-bis(3-methoxyphenyl)-3-methylpiperidin-4-one oxime (3h, C₂₇H₃₀N₂O₃)*: Off-white; Yield: 79%; m.p.: 128–130°C; ¹H NMR (400 MHz, CDCl₃): δ = 7.26 (t, *J* = 6.9 Hz, 3H), 7.11–7.04 (m, 6H), 6.88–6.85 (m, 2H), 6.81–6.77 (m, 2H), 3.82 (s, 3H), 3.81 (s, 3H), 3.68 (dd, *J* = 2.7, 8.6 Hz, H-6a), 3.60 (d, *J* = 14.6 Hz, 1H), 3.51–3.44 (m, 2H), 3.32 (d, *J* = 10.24 Hz, H-3), 2.59–2.51 (m, H-5a), 2.17 (q, H-5e) 0.77 (d, *J* = 6.6 Hz, CH₃ at C-3) ppm; ¹³C NMR (100.52 MHz, CDCl₃): δ = 159.92 (C-4), 159.77 (C-6'c), 159.55 (C-2'c), 145.71 (C-6'), 144.05 (C-2'), 136.90 (N-Bn *ipso* carbon), 130.03, 129.69, 129.30, 127.56, 126.60, 121.79, 120.24, 114.64, 113, 112.71, 112.62, 72.52 (C-2), 64.04 (C-6), 55.33 (2 O- $\underline{\text{CH}_3}$), 53.82 (N- $\underline{\text{CH}_2}$ -Ph), 43.67 (C-3), 34.80 (C-5), 12.81 ($\underline{\text{CH}_3}$ at C-3) ppm.

2.9i *1-Benzyl-3-ethyl-2,6-bis(4-isopropylphenyl)piperidin-4-one oxime (3i, C₃₂H₄₀N₂O)*: White solid; Yield: 82%; m.p.: 180–182°C; ¹H NMR (400 MHz, CDCl₃): δ = 8.16 (s, OH), 7.39–7.34 (m, 4H), 7.15–7.12 (m, 4H), 7.05–7.04 (m, 3H), 6.82–6.80 (m, 2H), 3.72 (dd, *J* = 3.3, 11.3 Hz, H-6a), 3.54–3.38 (m, 4H), 2.90–2.84 (m, 2H), 2.45–2.40 (m, 1H), 2.24–2.17 (m, H-5e), 1.46–1.39 (m, 1H), 1.35–1.23 (m, 13H), 0.76 (t, *J* = 7.5 Hz, 3H) ppm; ¹³C NMR (100.52 MHz, CDCl₃): δ = 158.52 (C-4), 147.92 (C-6'c), 147.88 (C-2'c), 141.47 (C-6'), 140.01 (C-2'), 138.12 (N-Bn *ipso* carbon), 129.70, 128.95, 127.84, 127.49, 126.56, 126.58, 70.74 (C-2), 64.40 (C-6), 54.54 (C-3), 50.62 (N- $\underline{\text{CH}_2}$ -Ph), 35.24 (C-5), 33.90 ($\underline{\text{CH}}$ of *iPr* at C-6'c, C-2'c), 24.20 (2CH₃ at C-6'c), 24.13 (2 $\underline{\text{CH}_3}$ at C-2'c), 20.39 ($\underline{\text{CH}_2}$ -CH₃ at C-3), 12.87 (CH₃ at C-3) ppm.

2.9j *1-Benzyl-2,6-bis(4-chlorophenyl)-3-ethylpiperidin-4-one oxime (3j, C₂₆H₂₆N₂O)*: White solid; Yield: 86%; m.p.: 168–170°C; ¹H NMR (400 MHz, CDCl₃): δ = 7.42–7.37 (m, 4H), 7.29 (d, *J* = 8.4 Hz, 4H), 7.15–7.07 (m, 3H), 6.80–6.77 (m, 2H), 3.78 (dd, *J* = 3.6, 11.0 Hz, H-6a), 3.58–3.33 (m, 4H), 2.38–2.33 (m, H-5e), 2.17 (q, H-5e), 1.50–1.39 (m, 1H), 1.18–1.13 (m, 1H), 0.76 (t, *J* = 7.3 Hz, CH₃ at C-3) ppm; ¹³C NMR (100.52 MHz, CDCl₃): δ = 157.34 (C-4), 142.42 (C-6'c), 141.16 (C-2'c), 136.89 (N-Bn *ipso* carbon), 133.14 (C-6'), 133.01 (C-2'), 130.23, 129.62, 129.10, 128.91, 128.64, 127.83, 126.81, 69.75 (C-2), 63.37 (C-6), 54.46 (N- $\underline{\text{CH}_2}$ -Ph), 50.56 (C-3), 34.91 (C-5), 20.40 ($\underline{\text{CH}_2}$ at C-3), 12.04 ($\underline{\text{CH}_3}$ at C-3) ppm.

2.9k *1-Benzyl-2,6-bis(4-ethoxyphenyl)-3-ethylpiperidin-4-one oxime (3k, C₃₀H₃₆N₂O₃)*: White solid; Yield: 91%, m.p.: 148–150°C; ¹H NMR (400 MHz, CDCl₃): δ = 7.36 (q, 4H), 7.08 (unresolved triplet, 3H), 6.84 (d, *J* = 8.8 Hz, 6H), 4.05–3.99 (m, 4H), 3.72–3.67 (m, 1H), 3.55–3.37 (m, 4H), 2.39–2.35 (m, H-5a), 2.16 (t, *J* = 12.6 Hz, H-5e), 1.42–1.38 (m, 6H), 1.25–1.20 (m, 1H), 1.18–1.13 (m, 1H), 0.75 (t, *J* = 7.3 Hz, CH₃ of Ethyl at C-3) ppm; ¹³C NMR (100.52 MHz, CDCl₃): δ = 158.24 (C-4), 158.18 (C-6'c), 158.11 (C-2'c), 137.63 (C-6'), 136.08 (C-2'), 134.57 (N-Bn *ipso* carbon), 129.99, 129.76, 128.82, 127.52, 126.37, 114.48, 114.18, 70.06 (C-2), 63.57 (C-6), 53.72 (N- $\underline{\text{CH}_2}$ -Ph), 50.94 (C-3), 35.47 (C-5), 20.09 ($\underline{\text{CH}_3}\text{CH}_2\text{O}$ at C-6'c, C-2'c), 15.01 ($\underline{\text{CH}_2}\text{CH}_3$ at C-3), 12.10 ($\underline{\text{CH}_3}$ at C-3) ppm.

2.9l *1-Benzyl-3-isopropyl-2,6-diphenylpiperidin-4-one oxime (3l, C₂₇H₃₀N₂O)*: Brown semi-solid; Yield: 87%; ¹H NMR (400 MHz, CDCl₃): δ = 7.56 (d, *J* = 7.3 Hz, 2H), 7.39–7.35 (m, 4H), 7.28–7.24 (m, 3H), 7.18–7.09 (m, 4H), 6.93 (dd, *J* = 2.0, 6.5, 2H), 4.31–4.22 (m, 2H), 3.70 (d, *J* = 14.2 Hz, 1H), 3.55 (d, *J* = 14.2 Hz, 1H), 2.89–2.76 (m, 2H), 2.14 (dd, *J* = 3.1, 8.9 Hz, H-5e), 1.79–1.71 (m, 1H), 0.96 (d, *J* = 6.6 Hz, 3H), 0.83 (d, *J* = 6.6 Hz, 3H) ppm; ¹³C NMR (100.52 MHz, CDCl₃): δ = 157.66 (C-4), 145.58 (C-6'), 144.65 (C-2'), 137.87 (N-Bn *ipso* carbon), 129.66, 128.80, 128.21, 127.98, 127.89, 127.35, 62.83 (C-2), 60.01 (C-6), 56.15 (N- $\underline{\text{CH}_2}$ -Ph), 55.68 (C-3), 35.41 (C-5), 30.15 ($\underline{\text{CH}}$ of *iPr*), 21.72 ($\underline{\text{CH}_3}$ *iPr*) 20.63 ($\underline{\text{CH}_3}$ *iPr*) ppm.

2.9m *1-Benzyl-3,5-dimethyl-2,6-diphenylpiperidin-4-one oxime (3m, C₂₆H₂₈N₂O)*: Off-white solid; Yield: 94%; m.p.: 132–134°C; ¹H NMR (400 MHz, CDCl₃): δ = 7.42–7.40 (m, 4H), 7.34–7.11 (m, 9H), 6.96 (d, *J* = 7.3 Hz, 2H), 4.00 (d, *J* = 5.8 Hz, H-6a), 3.80 (d, *J* = 6.6 Hz, H-2a), 3.71 (d, *J* = 14.2 Hz, 1H), 3.58–3.55 (m, 2H), 2.62–2.55 (m, H-5a), 1.24 (d, *J* = 6.9 Hz, 3H), 0.98 (d, *J* = 7.3 Hz, 3H) ppm; ¹³C NMR (100.52 MHz, CDCl₃): δ = 163.05 (C-4), 144.57 (C-2'c, 6'c), 138.33 (N-Bn *ipso* carbon), 129.72, 128.48, 128.22, 127.90, 126.94, 126.78, 69.50 (C-2), 62.11 (C-6), 57.06 (N- $\underline{\text{CH}_2}$ -Ph), 41.87 (C-3), 38.48 (C-5), 17.67 ($\underline{\text{CH}_3}$ at C-3), 16.84 ($\underline{\text{CH}_3}$ at C-5) ppm.

2.9n *1-Benzyl-2,6-bis(4-isopropylphenyl)-3,5-dimethylpiperidin-4-one oxime (3n, C₃₂H₄₀N₂O)*: White solid; Yield: 92%; m.p.: 158–160°C; ¹H NMR (400 MHz, CDCl₃): δ = 7.31 (d, *J* = 8.0 Hz, 4H),

7.16–7.08 (m, 7H), 6.93 (d, $J = 6.9$ Hz, 2H), 3.92 (d, $J = 6.2$ Hz, H-6a), 3.79 (d, $J = 6.2$ Hz, H-2a), 3.69 (d, $J = 14.2$ Hz, 1H), 3.57–3.51 (m, 2H), 2.91–2.81 (m, two CH of *iPr*), 2.62–2.55 (m, H-5a), 1.25–1.21 (m, 15H of 2(CH₃)₂ merged with CH₃ at C-3), 1.00 (d, $J = 6.9$ Hz, 3H of CH₃ at C-5) ppm; ¹³C NMR (100.52 MHz, CDCl₃): $\delta = 163.05$ (C-4), 147.42 (C-6'c), 146.72 (C-2'c), 142.00 (C-6'), 141.61 (C-2'), 139.02 (N-Bn *ipso* carbon), 129.60, 128.28, 128.14, 127.78, 126.50, 126.40, 126.17, 69.74 (C-2), 65.62 (C-6), 56.95 (N-CH₂-Ph), 42.00 (C-3), 38.56 (C-5), 33.88 (2CH *iPr*), 33.78 (2CH *iPr*), 24.20 (4CH₃ *iPr*), 18.16 (CH₃ at C-3), 16.70 (CH₃ at C-5) ppm.

2.9o *1-Benzyl-2,6-bis(4-ethoxyphenyl)-3,5-dimethylpiperidin-4-one oxime (3o, C₃₀H₃₆N₂O₃)*: White solid; Yield: 88%; m.p.; 172–174°C; ¹H NMR (400 MHz, CDCl₃): $\delta = 8.28$ (s, OH), 7.28 (t, $J = 8.6$ Hz, 4H), 7.15–7.08 (m, 3H), 6.94 (d, $J = 5.8$ Hz, 2H), 6.85 (d, $J = 8.4$ Hz, 2H), 6.77 (d, $J = 8.8$ Hz, 2H), 4.05–3.96 (m, 4H), 3.91 (d, $J = 6.2$ Hz, H-6a), 3.78 (d, $J = 6.9$ Hz, H-2a), 3.69 (d, $J = 14.3$ Hz, 1H), 3.55–3.49 (m, 2H), 2.59–2.52 (m, H-5a), 1.42–1.37 (m, 6H of 2CH₃CH₂O), 1.22 (d, $J = 7.3$ Hz, 3H of CH₃ at C-3), 0.97 (d, $J = 6.9$ Hz, 3H of CH₃ at C-3) ppm; ¹³C NMR (100.52 MHz, CDCl₃): $\delta = 163.45$ (C-4), 158.19 (C-6'c), 157.86 (C-2'c), 138.95 (C-6'), 136.62 (C-2'), 136.39 (N-Bn *ipso* carbon), 129.61, 129.22, 127.83, 126.60, 69.27 (C-2), 64.87 (C-6), 63.48 (OCH₂), 63.44 (OCH₂), 56.81 (N-CH₂-Ph), 42.06 (C-3), 38.57 (C-5), 17.77 (CH₃ at C-3), 16.77 (CH₃ at C-5), 15.04 (CH₃CH₂O at C-6'c, C-2'c) ppm.

3. Results and discussion

3.1 Chemistry

The highly functionalized 1-benzyl-3,5-dialkyl-2,6-diarylpiperidin-4-one oximes **3a–o** were synthesized using appropriate piperidin-4-ones,^{18,19} sodium acetate trihydrate and hydroxylamine hydrochloride in ethanol as shown in scheme 1. It is interesting to note that it took only 1–2 h for the *i*-Pr and Cl-phenyl containing N-benzylpiperidin-4-ones to be converted to oximes, while the reaction time for the alkoxy-phenyl containing N-benzylpiperidin-4-ones was much longer, about 5 h. The synthesized compounds were characterized by ¹H/¹³C NMR and single-crystal X-ray diffraction studies. All the synthesized oximes from unsymmetrical ketones existed as E-isomer as witnessed by their NMR and XRD data.

Single-crystal X-ray diffraction analysis has been performed representatively for the 3-methyl and 3,5-dimethyl compounds **3d** and **3n**. The puckering parameters of the compounds were analysed according to Cremer and Pople and Nardelli.^{14,15} For the piperidone ring C1-C2-C3-C4-C5-N1 of **3d**, the smallest displacement asymmetry parameters q_2 and q_3 are 0.0911 and -0.5598 Å, respectively. The ring puckering parameters such as total puckering amplitude ' Q_T ' and phase angle ' θ ' are 0.5670 Å and 169.37°. Thus, all puckering parameters strongly support a slightly distorted chair conformation for the piperidone ring.

The stereochemistry of the substituents are analysed as follows. The equatorial orientation of the methyl group is witnessed by its torsion angles 177.4° [C2-C3-C4-C12] and 176.9° [N1-C1-C2-C12]. The torsion angles of the phenyl groups on both sides of the amino group are 171.8° [C3-C2-C1-C6] and -178.4° [C3-C4-C5-C13], which support their equatorial orientation. The 'N' atom of the piperidone molecule shows the sp³ hybridization, which can be evidenced from the angles around that nitrogen. The N-benzyl group also adopts an equatorial disposition to the best plane of the piperidone ring, which is evidenced from the torsion angles C19-N1-C5-C4 = 178.4° and C19-N1-C1-C2 = -176.9°. X-ray analysis of **3d** revealed the conformation of the molecule with C1, C2 and C5 having R, R and S relative configurations, respectively. Overall, the detailed crystallographic studies such as asymmetry parameters, ring puckering parameters and torsion angles calculated for **3d** proved that the piperidone ring exists in a slightly distorted chair conformation with an equatorial orientation of the methyl group on one of the active methylene centres and phenyl rings on both sides of the tertiary amino group along with an equatorial orientation of the benzyl group on the tertiary amino group. ORTEP of the compound **3d** is shown in figure 2.

Single-crystal analysis of **3n** clearly indicates that the molecule exist in a twist-boat conformation with R, R, S and S relative configurations of C1, C2, C4 and C5, respectively. The ring puckering parameters ' Q_T ' and ' θ ' are 0.7687 Å and 89.38°. ORTEP of the compound **3n** is shown in figure 3.^{21,22}

3.2 Cytotoxicity

All the synthesized compounds **3a–o** were subjected to MTT assay to evaluate *in vitro* cytotoxicity against HeLa cells.²³ The IC₅₀ values of all compounds are summarized along with their structure in table 1 for better structure activity comprehension. Besides, the standard drugs camptothecin and etoposide,^{24–27} were

Scheme 1. Reagents and conditions: (a) Ethanol, warm. (b) Benzyl bromide, anhydrous K_2CO_3 , THF, rt, 72 h. (c) Hydroxylamine hydrochloride, sodium acetate trihydrate, ethanol, reflux, 1–5 h.

Figure 2. The ORTEP of compound **3d**. Ellipsoids are drawn at 50% probability. Crystal analysis clearly proved that the oxime adopts the *E* configuration.

also analysed under identical conditions and their IC_{50} values are also reproduced in the table 1.

Analyses of the cytotoxic data from table 1 project some lead molecules. All the tested compounds **3a–o** exhibited good antiproliferative activity against the cancer cells with IC_{50} values ranging from 13.88 to 37.94 μM concentration except compound **3g**, which required 93.91 μM . Among them, compounds **3a**, **3l**

Figure 3. The ORTEP of compound **3n**. Ellipsoids are drawn at 40% probability. One of the methyl atoms C25 is disordered in five different positions with 0.2 occupancy. The C25 atom has been refined isotropically and no hydrogen atoms were fixed for this atom.

and **3m** with unsubstituted phenyl groups on both sides of the tertiary amino group have shown lesser activity (25.23, 34.63 and 37.94 μM , respectively) than their

Table 1. ^aCytotoxic effect of compounds **3a–o** on HeLa cells.

Compound	Structure	^b Linear regression equation ^a [log]: $Y=A+Bx$	<i>R</i> value	^c IC ₅₀ in μM
3a		$Y = 9.97629 + (41.34597)x$	0.98	25.23 ± 3.8
3b		$Y = -0.90241 + (50.11737)x$	0.93	25.98 ± 2.04
3c		$Y = 12.19751 + (47.24709)x$	0.98	13.88 ± 0.93
3d		$Y = 4.93121 + (52.61212)x$	0.97	16.39 ± 2.47
3e		$Y = 8.459 + (43.24166)x$	0.98	21.21 ± 1.42
3f		$Y = 17.6655 + (37.30817)x$	0.98	16.10 ± 3.06
3g		$Y = -35.01759 + (55.23339)x$	0.96	93.91 ± 3.38
3h		$Y = -17.78817 + (57.67985)x$	0.94	32.17 ± 2.37

Table 1. (contd.)

Compound	Structure	^b Linear regression equation ^a [log]: $Y=A+Bx$	<i>R</i> value	^c IC ₅₀ in μ M
3i		$Y = 0.59467 + (47.01865)x$	0.95	23.60 ± 1.20
3j		$Y = -5.928 + (56.84707)x$	0.95	21.28 ± 2.13
3k		$Y = 3.46962 + (45.80483)x$	0.98	22.07 ± 3.4
3l		$Y = -29.78378 + (67.83093)x$	0.94	34.63 ± 1.43
3m		$Y = -32.48966 + (68.4888)x$	0.95	37.94 ± 1.32
3n		$Y = 7.52381 + (48.38217)x$	0.99	16.08 ± 1.43
3o		$Y = 10.47483 + (44.62101)x$	0.96	16.23 ± 1.65
Etoposide		$Y = 2.27708 + (41.80905)x$	0.98	23.33 ± 0.92
Camptothecin		$Y = 29.02966 + (42.56504)x$	0.99	8.93 ± 0.54

^aExponentially growing cells were treated with different concentrations of test compounds for 24 h and cell growth inhibition was analysed through MTT assay

^bStructure drawn on the basis of found relative configuration using Single-crystal XRD analysis (**3d** and **3n**) and 1D NMR

^cMean percent decrease in cell number of five independent experiments was used to calculate the linear regression equation. Linear regression: $Y = A + Bx$ ($A = Y$ -intercept; $B =$ slope of the line; $x = x$ -scale)

^dIC₅₀ is defined as the concentration, which results in a 50% decrease in cell number as compared with that of the control cultures in the absence of an inhibitor. The values represent the mean $\pm$ SD of five individual observations

analogues halo/alkyl/alkoxy substituted phenyl compounds. Compounds **3c**, **3d** and **3f** with isopropyl, ethoxy and chloro substituents on the phenyl groups at C-2/C-6 along with the methyl at C-3 showed their best activity, below the IC_{50} of 16.39 μ M. Of them, particularly compound **3c**, the *para*-iPr-phenyl compound registered the best activity in this series, which inhibited the proliferation of cancer cells with an IC_{50} of 13.88 μ M.

In addition to the methyl group at C-3, one more methyl group was introduced on another active methylene centre at C-5. On the other hand, to improve the efficacy, we replaced the methyl group at C-3 by the ethyl and isopropyl groups. However, either the replacement of methyl at C-3 by ethyl and isopropyl or incorporation of another methyl at C-5 did not improve the efficacy of compounds **3c**, **3d** and **3f**. Instead, the 3-ethylated analogues (**3i**, **3j** and **3k**, respectively) of **3c**, **3d** and **3f** required the IC_{50} between 21.28 and 23.60 μ M, and the 3,5-dimethylated analogs **3n** (16.08 μ M) and **3o** (16.23 μ M) exhibited a similar range of cytotoxicity. Overall, many compounds *viz.*, **3c–f**, **3j–k** and **3n–o** are better than one of the standard drugs Etoposide (23.33 μ M) and **3c** is comparable to another anticancer drug camptothecin (8.93 μ M) under identical conditions. As a reference, MTT assay was also performed with lead test compounds using normal healthy PBMCs (peripheral blood mononuclear

cell). There was no significant indication of cell death in these cells even at higher concentration (100 μ M) of the test compounds. This confirmation of non-toxicity on healthy cells can be a chemotherapeutic measure to propose these compounds as medicinally important drug leads. The IC_{50} (inhibition of cell viability to 50%) concentrations were calculated using the respective regression equation as shown in table 1 and figure 4.

3.3 Altered morphology study

The altered morphology of exposed cells (1×10^5 cells/well) at IC_{50} concentration was studied after 24 h using phase contrast microscope (DMI6000B, Leica Microsystems, Wetzlar, Germany) and manifested in the figure 5.

The altered morphology of the cells as a rejoinder to the effect of test compounds was observed under a phase-contrast microscope. The group that lacks the test compound was considered as control. As depicted in figure 5a, all the control cells have shown normal healthy and intact nuclei without any cytological abnormalities (figure 5a). The remaining cells were treated with IC_{50} concentration of highly toxic test compound **3c** for 24 h. Number of cytomorphological anomalies were observed in the test group *viz.*, blebbing of cellular membrane, chromatin condensation, fragmentation

Figure 4. The graphs of linear regression analysis representing the growth inhibition of different test compounds with IC_{50} values below 20 μ M.

Figure 5. Light and fluorescent micrographs of normal and treated HeLa cells. Phase contrast micrographs of normal and treated (with the IC_{50} concentration of ideal compound **3c** for 24 h) HeLa cells (**a** and **b**, respectively). Fluorescence micrographs of normal and treated (with the IC_{50} concentration of ideal compound **3c** for 24 h) HeLa cells with Hoechst 33342 stain (**c** and **d**, respectively) and Propidium Iodide (**e** and **f**, respectively). The stained cells were detected by fluorescence light microscope at 360/470 nm and 535/617 nm excitation/emission for Hoechst stain and PI, respectively. Each inset represents the magnified image of the corresponding normal and deformed cell/nuclei.

and formation of apoptotic bodies (figure 5b). Most of the treated cells exhibited the symptoms of apoptosis but the damage was intense in some cells, with the cell membrane rupture and the subsequent release of cytoplasm as observed in figure 5b (inset). To confirm the light microscopy data and to visualize dead cells in a more proper manner, the cells were exposed to fluorescence microscopy using Hoechst 33342 and PI stains for control and treated cells (figure 5c and d, respectively). Obviously, the bright condensed chromatin was identified, leading to the deformed nuclear cytoplasmic consistency followed by the margination of chromatin into a horseshoe shaped structure which was a clear indication of early apoptosis (figure 5d, inset) for the

cells treated with the same concentration as described above. The destructive fragmentation of the nucleus (karyorrhexis) resulted in the picknosis of the treated cells.^{27,28} When stained with PI, the dead cells took up the stain to give a clear quantitative picture of the cytotoxic effect of the test compound at its IC_{50} concentration. It is noteworthy here that only the dead cells take up PI staining thus allowing a better visual analysis of the toxicity of the test compound.

3.4 Annexin V-PI dual staining to access apoptosis

Fluorescence microscopy is a wonderful tool to access cellular processes and the corresponding morphological

Figure 6. Annexin V-FITC/PI dual staining to access the degree of apoptosis. Phase contrast (a–d) and fluorescence (e–h) micrographs of control and **3c** (13.88 μ M) treated HeLa cells.

changes occurring in the cells in response to external stimuli. HeLa cells untreated and treated with 13.88 μ M lead compound **3C** were stained with Annexin V labelled with FITC and counter stained with PI after different time intervals (0, 4, 12, 24 h). Annexin V specifically binds to the phosphatidylserine in the cells, marking its relocation to the outer-cell membrane and PI enters only dead cells as it cannot pass through the intact cell membrane. Dual staining with Annexin V-FITC and PI simultaneously, gives a clear indication of early/late apoptosis (with exposure of phosphatidylserine) and cell death. Our results showed marked apoptosis of **3C** treated HeLa cells in a time dependent manner (figure 6). Early apoptosis after 4 h of treatment is depicted in figure 6f when the cells are stained with FITC alone whereas late apoptosis (12 h post treatment) marked the staining with both green FITC and red PI thus giving a yellowish (figure 6g). However, at the end of 24 h, a large number of PI stained cells with red fluorescence were observed indicating cell death (figure 6h).

4. Conclusion

All the synthesized novel N-benzylpiperidin-4-one oxime molecules were screened for their anticancer activity against HeLa cells. This effort has led to the identification of five new compounds **3c**, **3d**, **3f**, **3n** and **3o** with effective IC_{50} of 13.88 to 16.39 μ M. Of them, 1-benzyl-2,6-bis(4-isopropylphenyl)-3-methylpiperidin-4-one oxime **3c** with an IC_{50} of 13.88 μ M was found to be the best active compound.

Supplementary information

Crystallographic data of compounds **3d** (CCDC No. 841850) and **3n** (CCDC No. 841849) are provided as supplementary material. Supplementary crystallographic data for **3d** and **3n** can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html.

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