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Synthesis and antiproliferative evaluation of thiophene-coupled azetidin-3-ylamino quinoline-3-carbonitrile derivatives

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ABSTRACT

A novel series of (substituted)-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile derivatives (5a–5m) was synthesized from substituted 2-aminobenzoic acids (1a–1g) via 1H-benzo[d][1,3]oxazine-2,4-diones (2a–2g). A straightforward route was established for the synthesis of structurally varied quinoline-3-carbonitrile derivatives linked to thiophene-bearing azetidinyllamine units through halogenation, cyclization, displacement, S_NAr substitution, amide coupling, and Suzuki coupling. The structures of the final compounds were characterized by ¹H NMR, mass spectrometry, and elemental analysis, and their purity was assessed by HPLC. All synthesized compounds were evaluated for antiproliferative activity against A-549, DU-145, HeLa, and MCF-7 cancer cell lines using the MTT assay, with doxorubicin as the positive control. The most active synthesized compounds showed IC₅₀ values of 3.16–11.40 μM. Overall, compounds 5b, 5d, 5l, and 5m showed the most favorable activity profiles across the tested cell-line panel, although they were less potent than doxorubicin. Substitution at the 6- and 7-positions of the quinoline-3-carbonitrile scaffold influenced the observed antiproliferative activity.

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1. Introduction

Cancer comprises a heterogeneous group of diseases characterized by the uncontrolled growth of abnormal cells, which may invade adjacent tissues and spread to distant organs through metastasis. Its development is a multistep process influenced by genetic and environmental factors, including ultraviolet radiation, tobacco smoke, alcohol consumption, and infections caused by certain viruses or bacteria. Because early-stage disease may produce few or nonspecific symptoms, early detection remains important for improving treatment outcomes [1,2].

According to reports from the Indian Council of Medical Research (ICMR) and its National Cancer Registry Programme, approximately 1.57 million cancer cases were projected for 2025 in India, representing a 12.8% increase from 2020 [3]. Lung and oral cancers are among the common cancers in men, whereas breast, cervical, and ovarian cancers are common in women. Globally, cancer caused nearly 10 million deaths in 2020, with breast, lung, colorectal, and prostate cancers among the most frequently diagnosed types [4]. The emergence of drug resistance during prolonged treatment underscores the need for new antiproliferative agents with improved efficacy and safety. Heterocyclic scaffolds provide important structural

frameworks for anticancer drug discovery. In this study, quinoline-3-carbonitrile, azetidine, and thiophene pharmacophores were combined in a single molecular framework and evaluated against a panel of cancer cell lines. Quinoline derivatives [5] display diverse pharmacological properties and have been investigated as antitubercular [6], antimalarial [7], antibacterial [8], anti-HIV [9], and antitumor agents [10,11].

Thiophene is a five-membered sulfur-containing heterocycle widely used as a building block in medicinal chemistry, although its metabolism may generate reactive intermediates associated with drug-induced hepatotoxicity [12]. Thiophene derivatives can also function as C4 building blocks in the construction of complex ring systems and occur in compounds with diverse biological activities [13,14]. Azetidine is a four-membered nitrogen-containing ring found in several approved drugs, including baricitinib and cobimetinib [15–17]. Substituted 1H-benzo[d][1,3]oxazine-2,4-diones have also been investigated for antitumor, antipsychotic, antimycobacterial, antiviral, butyrylcholinesterase-inhibitory, and histone acetyltransferase-inhibitory activities [18–22]. Imidazole- and triazole-thione-based derivatives have likewise been reported as anticancer agents [23–25]. These observations support the investigation of hybrid structures incorporating quinoline-3-carbonitrile, thiophene, and azetidine motifs.

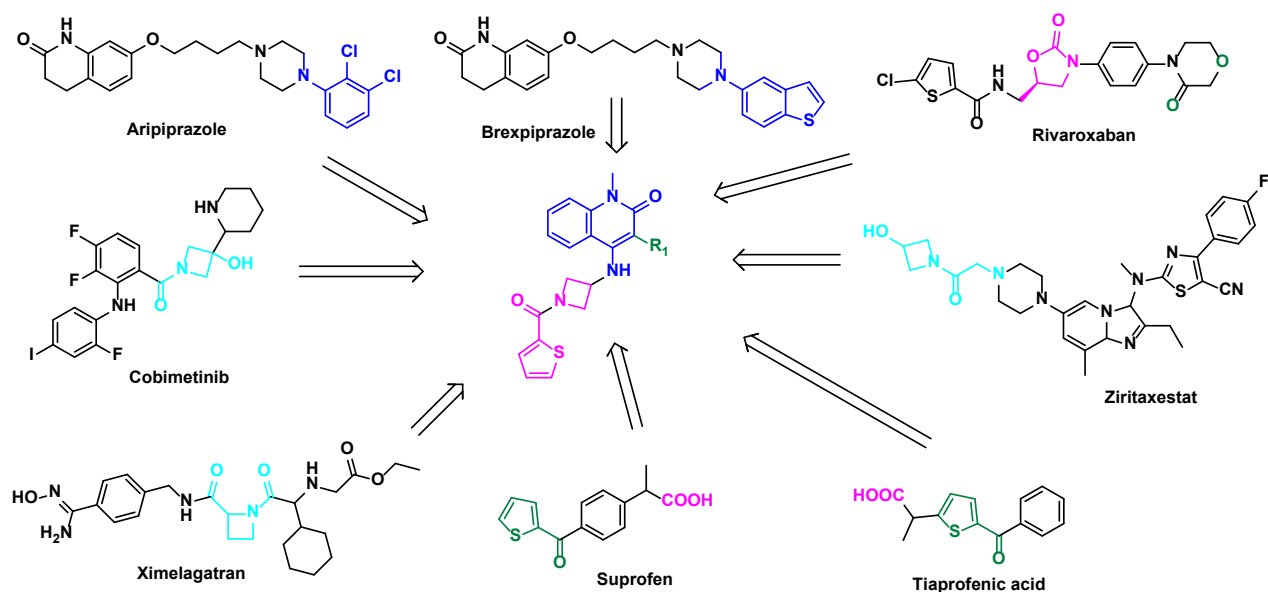


Figure 1. Representative drugs and bioactive compounds containing quinoline, thiophene, and/or azetidine structural motifs.

Molecular hybridization is a recognized strategy for designing new derivatives by combining pharmacologically relevant structural motifs. In the present work, literature precedents and marketed drugs containing quinoline, thiophene, or azetidine units were considered in the design of hybrid quinoline-3-carbonitrile derivatives bearing a thiophene-coupled azetidinyamine moiety (Figure 1).

Aripiprazole and brexpiprazole are antipsychotic agents used in the treatment of psychiatric disorders [26,27]. Vesnarinone is a quinolinone derivative investigated for its cardiogenic and antiproliferative effects [28]. Tiaprofenic acid and suprofen are thiophene-containing nonsteroidal anti-inflammatory agents, whereas rivaroxaban is an anticoagulant containing a thiophene-derived structural unit [29,30]. Cobimetinib is an azetidine-containing MEK1/2 inhibitor used in cancer therapy; other azetidine-containing agents include ziritaxestat and ximelagatran [31–33].

Marketed drugs containing thiophene-related structures include clopidogrel, ticlopidine, olanzapine, duloxetine, zileuton, cefoxitin, sertaconazole, and dorzolamide. Quinoline-containing drugs include chloroquine, primaquine, mefloquine, ciprofloxacin, moxifloxacin, topotecan, and bedaquiline [34–36]. Azetidine-containing drugs include baricitinib, cobimetinib, ezetimibe, azelnidipine, and siponimod [15–17,31,37]. These precedents illustrate the medicinal relevance of quinoline, thiophene, and azetidine frameworks and provided the rationale for combining these motifs in the present series [36]. Based on these literature precedents, quinoline, thiophene, and azetidine motifs were combined within a single molecular framework to investigate the antiproliferative activity of the resulting hybrid compounds.

2. Experimental

2.1. Materials and instrumentation

All chemicals were purchased from commercial suppliers (Sigma-Aldrich and Avra Labs) and used without further purification unless otherwise stated. Reaction progress was monitored by thin-layer chromatography (TLC) on Merck precoated silica gel 60 F₂₅₄ aluminum sheets, with visualization under UV light. Reactions were conducted under an inert atmosphere. Melting points were measured using a Casia-Siamia VMP-AM apparatus and are uncorrected. The final

compounds were characterized by ¹H NMR and LC-MS, and their purity was evaluated by HPLC. ¹H NMR spectra were recorded on a Varian NMR spectrometer at 400 MHz. Chemical shifts are reported in parts per million (δ , ppm), with the following abbreviations: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad signal (br). Mass spectra were recorded using a Micromass QUATTRO II instrument (Waters Corporation). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), fetal bovine serum (FBS), phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), and trypsin were obtained from Sigma-Aldrich. EDTA, glucose, and antibiotics were obtained from HiMedia Laboratories (Mumbai, India), and dimethyl sulfoxide (DMSO) was obtained from Merck (Mumbai, India).

2.2. Synthesis

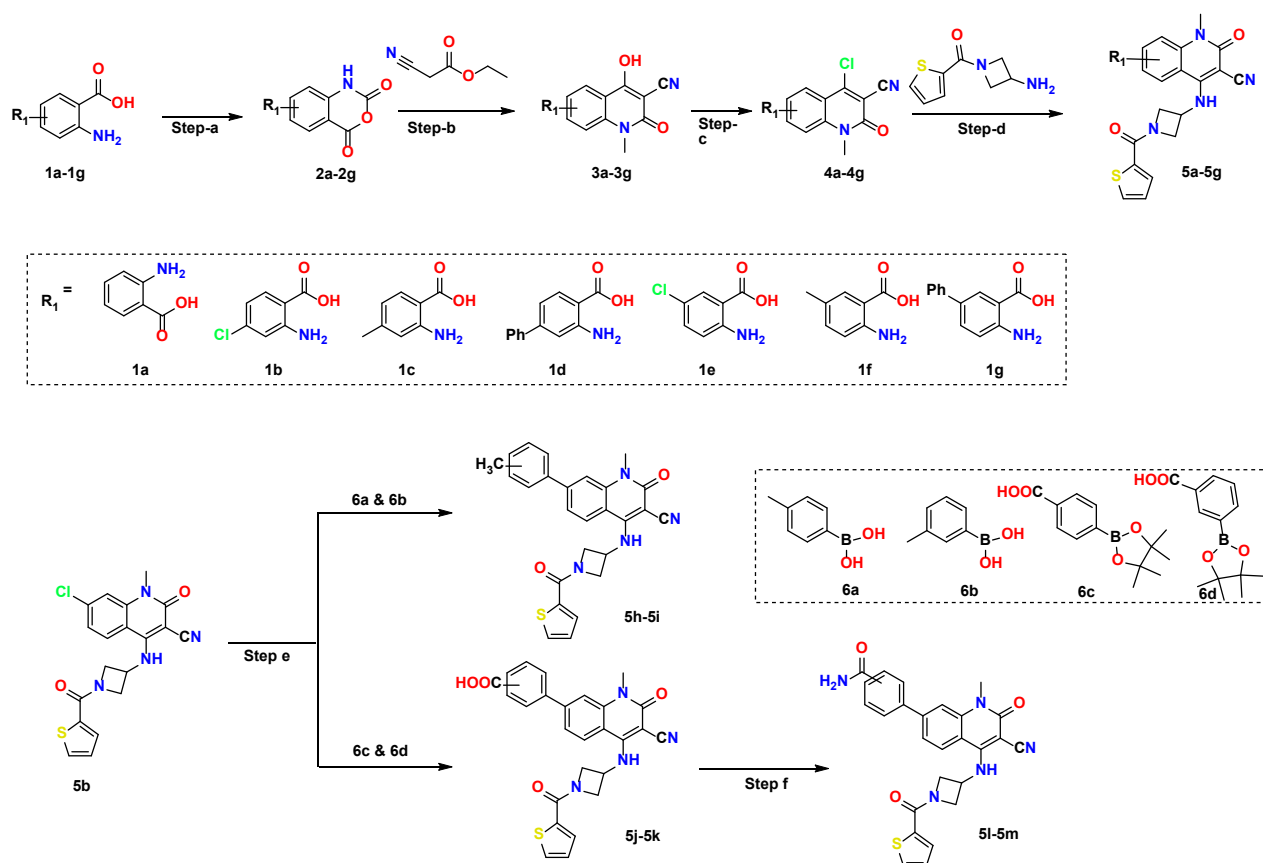
2.2.1. Step a: General procedure for the synthesis of (substituted)-1H-benzo[d][1,3]oxazine-2,4-dione (2a–2g)

To a stirred solution of 2-amino-(substituted)-benzoic acid derivatives (1a–1g, 1.0 eq.) in 1,4-dioxane (10 vol.) at 0 °C, triphosgene (2.0 eq.) was added portion wise under an argon atmosphere. The reaction mixture was then stirred to 85 °C for 16 h. Upon completion of the reaction (monitored by thin layer chromatography (TLC); 5:30 ethyl acetate/hexane, R_f = 0.1–0.4), the reaction mixture was poured into ice-cold water, resulting in precipitation of a solid product. The precipitate was collected by filtration, washed with water, and dried under vacuum to afford the corresponding (substituted)-1H-benzo[d][1,3]oxazine-2,4-dione derivatives (2a–2g) in 86–97% yield as solids (Scheme 1).

1H-Benzo[d][1,3]oxazine-2,4-dione (2a): Color: Yellow. Yield: 86%. ¹H NMR (400 MHz, DMSO-*d*₆, δ , ppm): 7.15 (d, J = 8.2 Hz, 1H, Ar-H), 7.28–7.22 (m, 1H, Ar-H), 7.77–7.72 (m, 1H, Ar-H), 7.91 (dd, J = 7.8, 1.4 Hz, 1H, Ar-H), 11.72 (s, 1H, NH).

7-Chloro-1H-benzo[d][1,3]oxazine-2,4-dione (2b): Color: Yellow. Yield: 96%. ¹H NMR (400 MHz, DMSO-*d*₆, δ , ppm): 7.86 (d, J = 2.0 Hz, 1H, Ar-H), 7.96 (dd, J = 8.4, 2.4 Hz, 1H, Ar-H), 8.13 (t, J = 10.4 Hz, 1H, Ar-H), 11.98 (s, 1H, NH).

7-Methyl-1H-benzo[d][1,3]oxazine-2,4-dione (2c): Color: Off-white. Yield: 96%. ¹H NMR (400 MHz, DMSO-*d*₆, δ , ppm): 2.33 (s, 3H, Ar-CH₃), 7.06 (d, J = 8.4 Hz, 1H, Ar-H), 7.58 (dd, J = 8.4, 1.4 Hz, 1H, Ar-H), 7.73 (s, 1H, Ar-H), 11.68 (s, 1H, NH).



Scheme 1. General scheme for the synthesis of (substituted)-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5a–5m). Reagents and conditions: (Step-a): Triphosgene, dioxane, 0 °C to RT and 85 °C, 16 h; (Step-b): NaH, DMF, MeI, 0 °C to RT, 2 h and ethyl 2-cyanoacetate, NaH, 0 °C to RT, 2 h; (Step-c): *N,N*-dimethylaniline, POCl₃, 90 °C, 2 h; (Step-d): (3-aminoazetidin-1-yl)(thiophen-2-yl)methanone, NaH, DMF, 0 °C to RT, 2 h; (Step-e): Aryl boronic acids and aryl boronate esters (6a–6d), Pd(PPh₃)₄, Na₂CO₃, dioxane, H₂O, 90 °C, 16 h; (Step-f): (15%) NH₃ in MeOH, EDC·HCl, HOBT, DMF, RT, 16 h.

7-Phenyl-1H-benzo[d][1,3]oxazine-2,4-dione (2d): Color: Pale yellow. Yield: 95%. ¹H NMR (400 MHz, DMSO-*d*₆, δ, ppm): 7.24 (d, *J* = 12.4 Hz, 1H, Ar-H), 7.39 (d, *J* = 9.2 Hz, 1H, Ar-H), 7.48 (d, *J* = 9.6 Hz, 2H, Ar-H), 7.71 (d, *J* = 9.6 Hz, 2H, Ar-H), 8.15–8.04 (m, 2H, Ar-H), 11.82 (s, 1H, NH).

6-Chloro-1H-benzo[d][1,3]oxazine-2,4-dione (2e): Color: Off-white. Yield: 88%. ¹H NMR (400 MHz, DMSO-*d*₆, δ, ppm): 7.16 (d, *J* = 8.8 Hz, 1H, Ar-H), 7.77 (dd, *J* = 8.8, 2.4 Hz, 1H, Ar-H), 7.88 (d, *J* = 2.4 Hz, 1H, Ar-H), 11.78 (s, 1H, NH).

6-Methyl-1H-benzo[d][1,3]oxazine-2,4-dione (2f): Color: Pale yellow. Yield: 94%. ¹H NMR (400 MHz, DMSO-*d*₆, δ, ppm): 2.33 (s, 3H, Ar-CH₃), 7.06 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.57 (dd, *J* = 8.4, 1.4 Hz, 1H, Ar-H), 7.72 (s, 1H, Ar-H), 11.68 (s, 1H, NH).

6-Phenyl-1H-benzo[d][1,3]oxazine-2,4-dione (2g): Color: Pale yellow. Yield: 97%. ¹H NMR (400 MHz, DMSO-*d*₆, δ, ppm): 7.24 (d, *J* = 12.4 Hz, 1H, Ar-H), 7.40 (d, *J* = 9.2 Hz, 1H, Ar-H), 7.50 (d, *J* = 9.6 Hz, 2H, Ar-H), 7.70 (d, *J* = 9.6 Hz, 2H, Ar-H), 8.16–8.04 (m, 2H, Ar-H), 11.84 (s, 1H, NH).

2.2.2. Step b: General procedure for the synthesis of (substituted)-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbonitrile (3a–3g)

Sodium hydride (NaH) (1.1 eq.) was added portion wise to a stirred solution of (substituted)-1H-benzo[d][1,3]oxazine-2,4-dione derivatives (2a–2g, 1.0 eq.) in dry dimethyl formamide (DMF, 10 vol.) at 0 °C under an argon atmosphere. The reaction mixture was stirred at room temperature for 30 min, then cooled again to 0 °C, and methyl iodide (1.05 eq.) was added drop wise. The mixture was gradually allowed to warm

to room temperature and stirred for 2 h. Subsequently, ethyl 2-cyano acetate (2.2 eq.) was added at 0 °C, followed by careful addition of sodium hydride (2.4 eq.). The reaction mixture was stirred at room temperature for 2 h, as monitored by TLC (10% methanol in dichloromethane, *R_f* = 0.1). The reaction was then quenched by cooling to 0 °C and adding ice-cold water. The resulting precipitate was collected by filtration, washed with water, and dried under vacuum to afford the corresponding (substituted)-4-hydroxy-1-methyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile derivatives (3a–3g) in 66–91% yield as solids (Scheme 1).

4-Hydroxy-1-methyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile (3a): Color: Yellow. Yield: 71%. ¹H NMR (400 MHz, DMSO-*d*₆, δ, ppm): 3.54 (s, 3H, Ar-NCH₃), 7.30 (t, *J* = 9.6 Hz, 1H, Ar-H), 7.53 (d, *J* = 11.6 Hz, 1H, Ar-H), 7.74 (t, *J* = 9.6 Hz, 1H, Ar-H), 8.08 (d, *J* = 10.8 Hz, 1H, Ar-H).

7-Chloro-4-hydroxy-1-methyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile (3b): Color: Off-white. Yield: 72%. ¹H NMR (400 MHz, DMSO-*d*₆, δ, ppm): 3.53 (s, 3H, Ar-NCH₃), 7.32 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.57 (s, 1H, Ar-H), 8.04 (d, *J* = 8.8 Hz, 1H, Ar-H).

4-Hydroxy-1,7-dimethyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile (3c): Color: Orange. Yield: 87%. ¹H NMR (400 MHz, DMSO-*d*₆, δ, ppm): 2.47 (s, 3H, Ar-CH₃), 3.54 (s, 3H, Ar-NCH₃), 7.16 (d, *J* = 10.4 Hz, 1H, Ar-H), 7.38 (s, 1H, Ar-H), 7.98 (d, *J* = 10.8 Hz, 1H, Ar-H).

4-Hydroxy-1-methyl-2-oxo-7-phenyl-1,2-dihydroquinoline-3-carbonitrile (3d): Color: Off-white. Yield: 68%. ¹H NMR (400 MHz, DMSO-*d*₆, δ, ppm): 3.66 (s, 3H, N-CH₃), 7.56–7.45 (m, 3H, Ar-H), 7.64 (dd, *J* = 10.8, 2.0 Hz, 1H, Ar-H), 7.71 (s, 1H, Ar-H), 7.87–7.84 (m, 2H, Ar-H), 8.17 (d, *J* = 9.2 Hz, 1H, Ar-H).

6-Chloro-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbonitrile (3e): Color: White. Yield: 80%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.63 (s, 3H, Ar-NCH $_3$), 7.76 (d, J = 12.4 Hz, 1H, Ar-H), 7.95 (dd, J = 11.8, 3.2 Hz, 1H, Ar-H), 8.04 (d, J = 2.8 Hz, 1H, Ar-H).

1,2-Dihydro-4-hydroxy-1,6-dimethyl-2-oxoquinoline-3-carbonitrile (3f): Color: Pale yellow. Yield: 91%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 2.38 (s, 3H, Ar-CH $_3$), 3.52 (s, 3H, Ar-NCH $_3$), 7.43 (d, J = 8.8 Hz, 1H, Ar-H), 7.57 (dd, J = 8.8, 1.6 Hz, 1H, Ar-H), 7.89 (s, 1H, Ar-H).

4-Hydroxy-1-methyl-2-oxo-6-phenyl-1,2-dihydroquinoline-3-carbonitrile (3g): Color: Off-white. Yield: 66%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.66 (s, 3H, N-CH $_3$), 7.42–7.39 (m, 1H, Ar-H), 7.50 (t, J = 9.6 Hz, 2H, Ar-H), 7.60 (d, J = 11.6 Hz, 1H, Ar-H), 7.73 (m, 2H, Ar-H), 8.04 (m, 1H, Ar-H), 8.26 (m, 1H, Ar-H).

2.2.3. Step c: General procedure for the synthesis of (substituted)-4-chloro-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbonitrile (4a–4g)

A mixture of (substituted)-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbonitrile derivatives (3a–3g, 1.0 eq.), *N,N*-dimethylaniline (1.0 eq.), and phosphorus oxychloride (POCl $_3$) (10 vol.) was gradually heated to 90 °C and stirred for 2 h. After completion of the reaction (monitored by TLC; 10% methanol in dichloromethane, R_f = 0.5), the volatile components were removed under reduced pressure to afford a sticky residue. The residue was quenched with ice-cold water, leading to precipitation of a solid product, which was collected by filtration, washed with water, and dried under vacuum to give the corresponding (substituted)-4-chloro-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbonitrile derivatives (4a–4g) in 78–92% yield as solids (Scheme 1).

4-Chloro-1-methyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile (4a): Color: Pale yellow. Yield: 91%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.67 (s, 3H, Ar-NCH $_3$), 7.51 (t, J = 9.6 Hz, 1H, Ar-H), 7.74 (d, J = 12.0 Hz, 1H, Ar-H), 7.95–7.90 (dt, J = 10.2, 2.4 Hz, 1H, Ar-H), 8.11–8.08 (m, 1H, Ar-H).

4, 7-Dichloro-1-methyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile (4b): Color: Pale yellow. Yield: 84%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.65 (s, 3H, Ar-NCH $_3$), 7.55 (dd, J = 11.8, 2.4 Hz, 1H, Ar-H), 7.86 (d, J = 2.4 Hz, 1H, Ar-H), 8.83 (d, J = 12.0 Hz, 1H, Ar-H).

4-Chloro-1, 7-dimethyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile (4c): Color: Pale yellow. Yield: 78%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 2.53 (s, 3H, Ar-CH $_3$), 3.65 (s, 3H, Ar-NCH $_3$), 7.34 (d, J = 11.2 Hz, 1H, Ar-H), 7.58 (s, 1H, Ar-H), 7.96 (d, J = 11.2 Hz, 1H, Ar-H).

4-Chloro-1-methyl-2-oxo-7-phenyl-1,2-dihydroquinoline-3-carbonitrile (4d): Color: Pale yellow. Yield: 84%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.77 (s, 3H, Ar-NCH $_3$), 7.62–7.47 (m, 3H, Ar-H), 7.94–7.78 (m, 4H, Ar-H), 8.12 (d, J = 11.2 Hz, 1H, Ar-H).

4, 6-Dichloro-1-methyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile (4e): Color: Yellow. Yield: 92%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.64 (s, 3H, Ar-NCH $_3$), 7.77 (d, J = 12.4 Hz, 1H, Ar-H), 7.96 (dd, J = 12.0, 3.2 Hz, 1H, Ar-H), 8.05 (d, J = 2.8 Hz, 1H, Ar-H).

4-Chloro-1,6-dimethyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile (4f): Color: Green. Yield: 92%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 2.54 (s, 3H, Ar-CH $_3$), 3.63 (s, 3H, Ar-NCH $_3$), 7.63 (d, J = 11.6 Hz, 1H, Ar-H), 7.18–7.74 (dd, J = 13.6, 2.0 Hz, 1H, Ar-H), 7.85 (s, 1H, Ar-H).

4-Chloro-1-methyl-2-oxo-6-phenyl-1, 2-dihydroquinoline-3-carbonitrile (4g): Color: Off-white. Yield: 84%. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.72 (s, 3H, Ar-NCH $_3$), 7.48–7.44 (m, 1H, Ar-H), 7.54 (t, J = 10.0 Hz, 2H, Ar-H), 7.88–7.78 (m, 3H, Ar-H), 8.27–8.22 (m, 2H, Ar-H).

2.2.4. Step d: General procedure for the synthesis of (substituted)-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5a–5g)

Sodium hydride (60% dispersion in mineral oil, 4.0 eq.) was added portion wise to a stirred solution of (substituted)-4-chloro-1-methyl-2-oxo-1, 2-dihydroquinoline-3-carbonitrile derivatives (4a–4g, 1.0 eq.) and (3-aminoazetidin-1-yl) (thiophen-2-yl)methanone (1.2 eq.) in dry DMF (10 vol.) at 0 °C under an argon atmosphere. The reaction mixture was stirred for 30 min at 0 °C and then allowed to warm gradually to room temperature, where it was further stirred for 2 h. Reaction progress was monitored by TLC (10% methanol in dichloromethane, R_f = 0.2). After completion, the reaction mixture was cooled to 0 °C, and crushed ice was added to quench the reaction. The resulting precipitate was collected by filtration, washed with water, and dried to afford the crude product, which was purified by combi-flash chromatography using a gradient of ethyl acetate in hexane (10–70%). This afforded the corresponding (substituted)-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile derivatives (5a–5g) in 78–90% yield as solids (Scheme 1).

1-Methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5a): Color: White. Yield: 78%. M.p.: 161–162 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.51 (s, 3H, N-CH $_3$), 4.16–4.05 (m, 4H, N-(CH $_2$) $_2$), 4.78–4.63 (m, 1H, NH-CH-(CH $_2$) $_2$), 7.37–7.27 (m, 2H, ArH), 7.49 (d, J = 8.31 Hz, 1H, ArH), 7.81–7.68 (m, 2H, ArH), 8.15–8.06 (m, 2H, ArH), 8.23 (d, J = 8.31 Hz, 1H, ArH). Anal. calcd. for C $_{19}$ H $_{16}$ N $_4$ O $_2$ S: C, 62.62; H, 4.43; N, 15.37. Found: C, 62.66; H, 4.36; N, 15.29 %. LC-MS (ES+): m/z 365.21 ($M+H^+$), R_t = 2.12 min, purity = 99.01%. HPLC (Isocratic; ACN:H $_2$ O, 80:20, 60 min; 0.1% TFA): R_t = 2.17 min, purity = 99.14%.

7-Chloro-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5b): Color: Off-white. Yield: 90%. M.p.: 177–178 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.34 (s, 3H, N-CH $_3$), 4.56–4.24 (m, 2H, N-CH $_2$), 4.93–4.60 (m, 2H, N-CH $_2$), 5.06 (br d, J = 6.24 Hz, 1H, NH-CH-(CH $_2$) $_2$), 7.20 (dd, J = 4.77, 4.03 Hz, 1H, Ar-H), 7.42 (dd, J = 8.80, 1.83 Hz, 1H, Ar-H), 7.65–7.48 (m, 2H, Ar-H), 7.93–7.65 (m, 1H, Ar-H), 8.48–8.17 (m, 2H, Ar-H). Anal. calcd. for C $_{19}$ H $_{15}$ ClN $_4$ O $_2$ S: C, 57.21; H, 3.79; N, 14.05. Found: C, 57.16; H, 3.84; N, 14.07 %. LC-MS (ES+): m/z 399.16 ($M+H^+$), R_t = 1.95 min, purity = 98.9%. HPLC (Isocratic; ACN:H $_2$ O, 80:20, 60 min; 0.1% TFA): R_t = 3.01 min, purity = 99.08%.

1, 7-Dimethyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5c): Color: Off-white. Yield: 83%. M.p.: 158–159 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 2.46 (s, 3H, Ar-CH $_3$), 3.52 (s, 3H, N-CH $_3$), 4.49–4.24 (m, 2H, N-CH $_2$), 4.92–4.62 (m, 2H, N-CH $_2$), 5.05 (br s, 1H, NH-CH-(CH $_2$) $_2$), 7.27–7.07 (m, 2H, Ar-H), 7.34 (s, 1H, Ar-H), 7.55 (br d, J = 3.67 Hz, 1H, Ar-H), 7.85 (d, J = 5.14 Hz, 1H, Ar-H), 8.31–8.03 (m, 2H, Ar-H). Anal. calcd. for C $_{20}$ H $_{18}$ N $_4$ O $_2$ S: C, 63.47; H, 4.79; N, 14.80. Found: C, 63.37; H, 4.84; N, 14.77 %. LC-MS (ES+): m/z 379.23 ($M+H^+$), R_t = 1.83 min, purity = 99.79%. HPLC (Isocratic; ACN:H $_2$ O, 80:20, 60 min; 0.1% TFA): R_t = 2.08 min, purity = 99.14%.

1-Methyl-2-oxo-7-phenyl-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5d): Color: Off-white. Yield: 88%. M.p.: 166–168 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.64 (s, 3H, N-CH $_3$), 4.57–4.27 (m, 2H, N-CH $_2$), 4.94–4.66 (m, 2H, N-CH $_2$), 5.10 (br s, 1H, NH-CH-(CH $_2$) $_2$), 7.33–7.08 (m, 1H, Ar-H), 7.62–7.40 (m, 4H, Ar-H), 7.73–7.61 (m, 2H, Ar-H), 8.02–7.74 (m, 3H, Ar-H), 8.47–8.13 (m, 2H, Ar-H). Anal. calcd. for C $_{25}$ H $_{20}$ N $_4$ O $_2$ S: C, 68.16; H, 4.58; N, 12.72. Found: C, 68.19; H, 4.51; N, 12.79 %. LC-MS (ES+): m/z 441.27 ($M+H^+$), R_t = 2.21 min, purity = 98.42%. HPLC (Isocratic; ACN:H $_2$ O, 80:20, 60 min; 0.1% TFA): R_t = 2.75 min, purity = 99.07%.

6-Chloro-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5e): Color: Off-white. Yield: 84%. M.p.: 181–183 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.50 (s, 3H, N-CH $_3$), 4.54–4.28 (m, 2H, N-CH $_2$), 4.92–4.62 (m, 2H, N-CH $_2$), 5.20–4.94 (m, 1H, NH-CH-(CH $_2$) $_2$), 7.40–7.07 (m, 1H, Ar-H), 7.65–7.41 (m, 2H, Ar-H), 7.76 (br d, J = 9.17 Hz, 1H, Ar-H), 7.85 (d, J = 5.14 Hz, 1H, Ar-H), 8.37–8.25 (m, 1H, Ar-H), 8.44 (d, J = 1.83 Hz, 1H, Ar-H). Anal. calcd. for C $_{19}$ H $_{15}$ ClN $_4$ O $_2$ S: C, 57.21; H, 3.79; N, 14.05. Found: C, 57.17; H, 3.83; N, 14.09 %. LC-MS (ES+): m/z 399.16 ($M+H^+$), R_t = 1.87 min, purity = 99.20%. HPLC (Isocratic; ACN:H $_2$ O, 80:20, 60 min; 0.1% TFA): R_t = 2.13 min, purity = 98.19%.

1,6-Dimethyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5f): Color: Yellow. Yield: 86%. M.p.: 149–150 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 2.40 (s, 3H, Ar-CH $_3$), 3.51 (s, 3H, N-CH $_3$), 4.57–4.18 (m, 2H, N-CH $_2$), 4.95–4.60 (m, 2H, N-CH $_2$), 5.07 (br s, 1H, NH-CH-(CH $_2$) $_2$), 7.25–7.14 (m, 1H, Ar-H), 7.41 (d, J = 8.80 Hz, 1H, Ar-H), 7.61–7.52 (m, 1H, Ar-H), 7.85 (d, J = 4.40 Hz, 1H, Ar-H), 8.11 (s, 1H, Ar-H), 8.21–8.19 (br d, J = 6.24 Hz, 2H, Ar-H and NH). Anal. calcd. for C $_{20}$ H $_{18}$ N $_4$ O $_2$ S: C, 63.47; H, 4.79; N, 14.80. Found: C, 63.53; H, 4.69; N, 14.87 %. LC-MS (ES+): m/z 379.23 ($M+H^+$), R_t = 1.82 min, purity = 99.32%. HPLC (Isocratic; ACN:H $_2$ O, 80:20, 60 min; 0.1% TFA): R_t = 2.96 min, purity = 99.02%.

1-Methyl-2-oxo-6-phenyl-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5g): Color: Off-white. Yield: 88%. M.p.: 172–173 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.57 (s, 3H, N-CH $_3$), 4.58–4.29 (m, 2H, N-CH $_2$), 4.95–4.67 (m, 2H, N-CH $_2$), 5.11 (br s, 1H, NH-CH-(CH $_2$) $_2$), 7.31–7.03 (m, 1H, Ar-H), 7.66–7.36 (m, 5H, Ar-H), 7.96–7.67 (m, 4H, Ar-H), 8.62–8.32 (m, 2H, Ar-H). Anal. calcd. for C $_{25}$ H $_{20}$ N $_4$ O $_2$ S: C, 68.16; H, 4.58; N, 12.72. Found: C, 68.11; H, 4.64; N, 12.83 %. LC-MS (ES+): m/z 441.21 ($M+H^+$), R_t = 2.17 min, purity = 98.72%. HPLC (Isocratic; ACN:H $_2$ O, 80:20, 60 min; 0.1% TFA): R_t = 3.47 min, purity = 97.72%.

2.2.5. Step e: General procedure for the synthesis of (substituted)-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5h–5k)

Arylboronic acids or aryl boronate esters (6a–6d, 1.5 equiv), Pd(PPh $_3$) $_4$ (0.05 equiv), and sodium carbonate (3.0 equiv) were added to a stirred solution of 7-chloro-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5b, 1.0 equiv) in 1,4-dioxane (20 vol.) and water (10 vol.). The mixture was degassed at room temperature for 15 min and then stirred at 90 °C for 16 h. Reaction progress was monitored by TLC (5% methanol in dichloromethane, R_f = 0.2). After completion, the mixture was cooled to room temperature and the solvents were removed under reduced pressure. For compounds 5h and 5i, the crude residue was diluted with water to afford a solid; for compounds 5j and 5k, the residue was treated with aqueous 1 N HCl (30 vol.) to afford a solid. The products were purified by silica-gel column chromatography (100–200 mesh), eluting with 2–5% MeOH in DCM. The appropriate fractions were combined and concentrated to afford compounds 5h–5k as off-white solids in 80–89% yield. The Suzuki coupling procedure was adapted from previously reported Pd(PPh $_3$) $_4$ /Na $_2$ CO $_3$ conditions in aqueous 1,4-dioxane [38] (Scheme 1).

1-Methyl-2-oxo-4-((1-(thiophene-2-carbonyl)azetid-3-ylamino)-7-(p-tolyl)-1,2-dihydroquinoline-3-carbonitrile (5h): Color: Off-white. Yield: 83%. M.p.: 167–168 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 2.42 (s, 3H, Ar-CH $_3$), 3.63 (s, 3H, N-CH $_3$), 4.56–4.24 (m, 2H, N-CH $_2$), 4.92–4.65 (m, 2H, N-CH $_2$), 5.10 (br d, 1H, J = 6.4 Hz, NH-CH-(CH $_2$) $_2$), 7.34–7.16 (m, 2H, Ar-H), 7.42 (t, J = 7.52 Hz, 1H, Ar-H), 7.71–7.54 (m, 5H, Ar-H), 8.07–7.76 (m, 1H, Ar-H), 8.46–8.15 (m, 2H, Ar-H). Anal. calcd. for C $_{26}$ H $_{22}$ N $_4$ O $_2$ S: C, 68.70; H, 4.88; N, 12.33. Found: C, 68.62; H, 4.89; N, 12.31 %.

LC-MS (ES+): m/z 455.10 ($M+H^+$), R_t = 2.29 min, purity = 96.92%. HPLC (Isocratic; ACN:H $_2$ O, 80:20, 60 min; 0.1% TFA): R_t = 2.89 min, purity = 99.68%.

1-Methyl-2-oxo-4-((1-(thiophene-2-carbonyl)azetid-3-ylamino)-7-(m-tolyl)-1,2-dihydroquinoline-3-carbonitrile (5i): Color: Off-white. Yield: 89%. M.p.: 171–173 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 2.42 (s, 3H, Ar-CH $_3$), 3.64 (s, 3H, N-CH $_3$), 4.59–4.25 (m, 2H, N-CH $_2$), 4.93–4.65 (m, 2H, N-CH $_2$), 5.10 (br d, J = 6.60 Hz, 1H, NH-CH-(CH $_2$) $_2$), 7.33–7.14 (m, 2H, Ar-H), 7.42 (t, J = 7.52 Hz, 1H, Ar-H), 7.74–7.54 (m, 5H, Ar-H), 8.07–7.74 (m, 1H, Ar-H), 8.46–8.15 (m, 2H, Ar-H). Anal. calcd. for C $_{26}$ H $_{22}$ N $_4$ O $_2$ S: C, 68.70; H, 4.88; N, 12.33. Found: C, 68.66; H, 4.92; N, 12.30 %. LC-MS (ES+): m/z 455.27 ($M+H^+$), R_t = 2.32 min, purity = 98.01%. HPLC (Isocratic; ACN:H $_2$ O, 80:20, 60 min; 0.1% TFA): R_t = 3.07 min, purity = 98.04%.

4-(3-Cyano-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinolin-7-yl)benzoic acid (5j): Color: Off-white. Yield: 80%. M.p.: 187–188 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.64 (s, 3H, N-CH $_3$), 4.49–4.22 (m, 2H, N-CH $_2$), 4.92–4.63 (m, 2H, N-CH $_2$), 5.12 (br s, 1H, NH-CH-(CH $_2$) $_2$), 7.20 (t, J = 5.6 Hz, 1H, Ar-H), 7.61–7.44 (m, 2H, Ar-H), 7.78–7.64 (m, 2H, Ar-H), 7.94–7.84 (m, 2H, Ar-H), 8.12–7.98 (m, 2H, Ar-H), 8.44–8.38 (m, 2H, Ar-H), 13.12 (br s, 1H, Ar-COOH). Anal. calcd. for C $_{26}$ H $_{20}$ N $_4$ O $_4$ S: C, 64.45; H, 4.16; N, 11.56. Found: C, 64.40; H, 4.09; N, 11.49 %. LC-MS (ES+): m/z 485.43 ($M+H^+$), R_t = 1.82 min, purity = 96.10%. HPLC (Isocratic; ACN:H $_2$ O 80:20, 60 min; 0.1% TFA): R_t = 2.88 min, purity = 97.04%.

3-(3-Cyano-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinolin-7-yl)benzoic acid (5k): Color: Off-white. Yield: 81%. M.p.: 191–192 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.65 (s, 3H, N-CH $_3$), 4.52–4.32 (m, 2H, N-CH $_2$), 4.93–4.69 (m, 2H, N-CH $_2$), 5.18–5.07 (br s, 1H, NH-CH-(CH $_2$) $_2$), 7.21 (t, J = 6.8 Hz, 1H, Ar-H), 7.56 (d, J = 5.2 Hz, 1H, Ar-H), 7.73–7.63 (m, 3H, Ar-H), 7.86 (d, J = 4.8 Hz, 1H, Ar-H), 8.08–7.94 (m, 1H, Ar-H), 8.22–8.10 (m, 1H, Ar-H), 8.47–8.32 (m, 3H, Ar-H), 13.10 (br s, 1H, Ar-COOH). Anal. calcd. for C $_{26}$ H $_{20}$ N $_4$ O $_4$ S: C, 64.45; H, 4.16; N, 11.56. Found: C, 64.41; H, 4.11; N, 11.47%. LC-MS (ES+): m/z 485.24 ($M+H^+$), R_t = 1.71 min, purity = 95.50%. HPLC (Isocratic; ACN:H $_2$ O 80:20, 60 min; 0.1% TFA): R_t = 2.57 min, purity = 99.01%.

2.2.6. Step f: General procedure for the synthesis of (substituted)-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinoline-3-carbonitrile (5l–5m)

HOBt (2.0 equiv) and EDC·HCl (2.0 equiv) were added under argon at 0 °C to a stirred suspension of 4- or 3-(3-cyano-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinolin-7-yl)benzoic acid (5j or 5k, 1.0 equiv) in DMF (10 vol.). A 15% solution of ammonia in methanol (10 vol.) was added at 0 °C, and the mixture was stirred at room temperature for 16 h. Reaction progress was monitored by TLC (5% methanol in dichloromethane, R_f = 0.2). After completion, the mixture was diluted with cold water. The resulting precipitate was collected by filtration, washed with water, and dried. The crude products were purified by silica-gel column chromatography (100–200 mesh), eluting with 2–5% MeOH in DCM. The appropriate fractions were combined and concentrated to afford compounds 5l and 5m as off-white solids in 82–85% yield. The EDC·HCl/HOBt-mediated amidation with methanolic ammonia was adapted from a reported procedure [39] (Scheme 1).

4-(3-Cyano-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetid-3-ylamino)-1,2-dihydroquinolin-7-yl)benzamide (5l): Color: Off-white. Yield: 82%. M.p.: 207–208 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 3.39 (s, 3H, N-CH $_3$), 4.45 (br s, 2H, N-CH $_2$), 4.83 (br d, J = 5.87 Hz, 2H, N-CH $_2$), 4.99 (br s, 1H, NH-CH-(CH $_2$) $_2$), 7.22–7.12 (m, 1H, Ar-H), 7.43 (br s, 2H, CONH $_2$), 7.54 (br d, J = 3.42 Hz, 1H, Ar-H), 7.82 (br d, J = 4.89 Hz, 1H, Ar-H),

7.90 (br s, 2H, Ar-H), 8.00 (br d, $J = 8.31$ Hz, 3H, Ar-H), 8.07 (br s, 1H, NH), 8.44–8.34 (m, 2H, Ar-H). Anal. calcd. for $C_{26}H_{21}N_5O_3S$: C, 64.58; H, 4.38; N, 14.48. Found: C, 64.64; H, 4.44; N, 14.53 %. LC-MS (ES⁺): m/z 484.38 ($M+H^+$), $R_t = 1.62$ min, purity = 98.50%. HPLC (Isocratic; ACN:H₂O, 80:20, 60 min; 0.1% TFA): $R_t = 1.58$ min, purity = 98.35%.

3-(3-Cyano-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1,2-dihydroquinolin-7-yl)benzamide (5m): Color: Off-white. Yield: 85%. M.p.: 217–219 °C. ¹H NMR (400 MHz, DMSO-*d*₆, δ , ppm): 3.66 (s, 3H, N-CH₃), 4.57–4.27 (m, 2H, N-CH₂), 4.98–4.64 (m, 2H, N-CH₂), 5.10 (br s, 1H, NH-CH(CH₂)₂), 7.37–7.07 (m, 1H, Ar-H), 7.77–7.47 (m, 5H, Ar-H), 7.89–7.78 (m, 1H, Ar-H), 8.11–7.90 (m, 2H, Ar-H), 8.17 (br s, 1H, Ar-H), 8.55–8.25 (m, 3H, Ar-H, CO-NH₂). Anal. calcd. for $C_{26}H_{21}N_5O_3S$: C, 64.58; H, 4.38; N, 14.48. Found: C, 64.65; H, 4.41; N, 14.52 %. LC-MS (ES⁺): m/z 484.31 ($M+H^+$), $R_t = 2.01$ min, purity = 98.75%. HPLC (Isocratic; ACN:H₂O, 80:20, 60 min; 0.1% TFA): $R_t = 2.64$ min, purity = 98.70%.

2.3. Biological evaluation

The synthesized compounds were evaluated for in vitro antiproliferative activity against the human lung cancer cell line A-549, prostate cancer cell line DU-145, cervical cancer cell line HeLa, and breast cancer cell line MCF-7. The cell lines were obtained from the National Centre for Cell Science (NCCS), Pune, India. The antiproliferative activity of compounds 5a–5m was evaluated in 96-well flat-bottom microplates using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric assay [40,41]. Stock cultures were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% heat-inactivated FBS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin in 75 cm² flasks at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were detached using TPVG solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS), counted with a hemocytometer, and diluted with the appropriate medium. Cell suspensions were prepared at 1×10^5 cells/mL and added to 96-well plates at 100 μ L/well. Test compounds were dissolved separately in DMSO and sterilized by filtration. To maintain a constant DMSO concentration across all treatment concentrations, serial dilutions of each test compound were first prepared in DMSO, and a fixed volume of each dilution was added to medium to give a constant final DMSO concentration of 0.1% (v/v) in every treatment well, irrespective of test-compound concentration. After overnight incubation, the medium was removed and replaced with 200 μ L of fresh medium containing the test compounds at 1–50 μ M. After 48 h, 20 μ L of MTT stock solution (5 mg/mL) was added to the 200 μ L treatment medium in each well, giving a final in-well MTT concentration of approximately 0.45 mg/mL in a total volume of 220 μ L. After incubation for 4 h, the MTT-containing medium was carefully removed, and the resulting formazan crystals were dissolved in 100 μ L DMSO. Absorbance was measured at 570 nm using a SpectraMax Plus microplate reader (Molecular Devices, CA, USA). IC₅₀ was defined as the concentration that produced 50% inhibition of cell growth.

Percentage inhibition was calculated using the following equation: % inhibition = $100 \times [1 - (\text{OD of the test compound} - \text{OD of the blank}) / (\text{OD of the vehicle control} - \text{OD of the blank})]$, where the vehicle control contained cells and medium with the same final DMSO concentration as the test wells but no test compound.

IC₅₀ values were obtained using GraphPad Prism (version 9.0), by fitting a four-parameter logistic (4PL) nonlinear regression model to the dose–response curves. SD was calculated across three independent experiments ($n = 3$), not the technical replicate wells. Doxorubicin was used as the reference anticancer agent. The final DMSO concentration in each assay well was 0.1% (v/v). Vehicle-control wells contained

cells and culture medium with the same final DMSO concentration (0.1% v/v) but no test compound; blank wells contained culture medium and MTT reagent without cells; and untreated-control wells contained cells and culture medium without DMSO or test compound. Doxorubicin was prepared as a 10 mM stock in DMSO and tested over a concentration range of 0.5–10 μ M. The assay was conducted in three independent experiments. Each concentration was tested in 3 replicate wells per concentration, and the complete experiment was independently repeated 3 times. The reported values represent the mean $\pm$ SD of three independent experiments ($n = 3$).

3. Results and discussion

3.1. Chemistry

The novel (substituted)-1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1, 2-dihydroquinoline-3-carbonitrile derivatives (5a–5m) were synthesized through a six-step sequence involving 1*H*-benzo[*d*][1,3]oxazine-2,4-dione as a key intermediate. Displacement and chlorination furnished the corresponding 4-chloro intermediates 4a–4g. Subsequent *S_NAr* substitution, Suzuki coupling, and amide coupling afforded the final compounds 5a–5m in good yields. The synthetic routes are shown in Scheme 1.

1*H*-Benzo[*d*][1,3]oxazine-2,4-dione derivatives have previously been synthesized using peroxy acids, peroxides, metal-catalyzed reactions, microwave-assisted methods, and multistep procedures [42]. In the present study, the synthesis of compounds 2a–2g was designed with consideration of reaction time, reaction conditions, number of steps, ease of isolation, scalability, and product purity. Treatment of substituted 2-aminobenzoic acids 1a–1g with triphosgene afforded the corresponding substituted 1*H*-benzo[*d*][1,3]oxazine-2,4-diones 2a–2g in 86–97% yields. Triphosgene is a thermally stable solid and provides a more convenient alternative to gaseous phosgene. In step a, the amino group is converted into a urea intermediate, followed by cyclization to afford compounds 2a–2g.

In step b, substituted 1,2-dihydro-4-hydroxy-1-methyl-2-oxoquinoline-3-carbonitriles 3a–3g were synthesized through a one-pot methylation/displacement sequence [43]. The substituted benzoxazine-2,4-diones were methylated with methyl iodide in the presence of sodium hydride in DMF. Additional sodium hydride and ethyl 2-cyanoacetate were then added to afford intermediates 3a–3g in 66–91% yields. In steps a and b, the products precipitated after the reaction mixtures were poured into cold water. The precipitates were collected and washed with excess cold water, ether, and pentane, thereby avoiding column chromatography.

In step c, chlorination of the 4-hydroxyquinoline intermediates afforded the corresponding 4-chloro derivatives 4a–4g, which served as key intermediates for the synthesis of the final compounds [44,45]. Compounds 5a–5g were obtained by displacement of the 4-chloro substituent through an *S_NAr* pathway. The adjacent nitrile group may facilitate this addition–elimination process by stabilizing the reaction intermediate. *S_NAr* conditions were screened to avoid the use of costly catalysts, as summarized in Table 1. The model reaction between 4-chloro-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbonitrile (4a) and (3-aminoazetidin-1-yl)(thiophen-2-yl)methanone was used to optimize the reaction. Sodium carbonate, potassium carbonate, cesium carbonate, sodium hydride, triethylamine, and *N,N*-diisopropylethylamine were evaluated as bases in dimethyl sulfoxide or dimethyl formamide. Reactions using most inorganic bases required heating, whereas the reaction with sodium hydride in DMF proceeded from 0 °C to room temperature within 2 h. Prolonged stirring at room temperature caused decomposition into unidentified side products.

Table 1. Optimization of reaction conditions for the S_NAr synthesis of compound 5a (step d).

Sr No	Condition	Yield (%)
1	K ₂ CO ₃ (3 eq), DMSO, 60 °C, 12 h	38
2	K ₂ CO ₃ (3 eq), DMF, 60 °C, 12 h	48
3	Na ₂ CO ₃ (3 eq), DMSO, 60 °C, 12 h	27
4	Na ₂ CO ₃ (3 eq), DMF, 60 °C, 12 h	34
5	Cs ₂ CO ₃ (3 eq), DMSO, 60 °C, 12 h	40
6	Cs ₂ CO ₃ (3 eq), DMF, 60 °C, 12 h	52
7	NaH (4 eq), DMSO, 0 °C to RT, 2 h	65
8	NaH (4 eq), DMF, 0 °C to RT, 2 h	78
9	TEA (5 eq), DMSO, 60 °C, 1 h	55
10	TEA (5 eq), DMF, 60 °C, 8 h	61
11	TEA (5 eq), NMP, 60 °C, 8 h	40
12	TEA (5 eq), ACN, 60 °C, 10 h	28
13	DIPEA (5 eq), DMSO, 60 °C, 12 h	27
14	DIPEA (5 eq), NMP, 60 °C, 12 h	38

Table 2. In vitro antiproliferative activity of the synthesized compounds against four cancer cell lines.

Compound	A-549 ^b	DU-145 ^c	HeLa ^d	MCF-7 ^e
5a	10.20±0.06	17.80±0.11	22.50±0.08	16.40±0.09
5b	4.14±0.04	5.24±0.06	3.16±0.13	4.29±0.04
5c	10.26±0.07	16.45±0.14	18.20±0.16	20.20±0.02
5d	4.25±0.09	4.26±0.04	4.37±0.08	5.35±0.13
5e	8.46±0.03	11.25±0.09	9.47±0.12	16.42±0.03
5f	15.70±0.08	22.60±0.13	26.80±0.12	31.42±0.06
5g	12.40±0.04	16.23±0.10	18.16±0.14	22.22±0.08
5h	7.34±0.14	12.43±0.15	8.21±0.06	14.23±0.09
5i	11.35±0.13	16.43±0.17	13.63±0.07	15.78±0.18
5j	11.25±0.18	8.12±0.08	10.16±0.09	22.15±0.12
5k	13.36±0.12	15.10±0.06	11.34±0.16	18.23±0.05
5l	5.82±0.09	7.35±0.17	3.65±0.06	4.16±0.17
5m	4.36±0.11	11.40±0.14	6.36±0.09	4.85±0.11
Doxorubicin (Reference)	1.92±0.06	1.82±0.12	1.75±0.08	2.16±0.11

^a Data are expressed as IC₅₀ (μM), mean ± SD; n = 3 independent experiments; SD: Standard deviation; IC₅₀: Half-maximal inhibitory concentration.

^b A-549: Human lung cancer cell line.

^c DU-145: Human prostate cancer cell line.

^d HeLa: Human cervical cancer cell line.

^e MCF-7: Human breast cancer cell line.

Reactions in DMSO generally afforded lower yields than those in DMF. Condition 8 afforded the highest yield and was selected for the synthesis of the remaining derivatives.

Derivatives 5h–5m were synthesized from compound 5b through Suzuki coupling followed, where applicable, by amide coupling. The general route is shown in Scheme 1. Suzuki coupling of 5b with p-tolylboronic acid (6a) or m-tolylboronic acid (6b) using Pd(PPh₃)₄ and sodium carbonate in dioxane afforded the corresponding tolyl derivatives. Coupling of 5b with 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoic acid (6c) and 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoic acid (6d) under basic conditions afforded the carboxylic acid derivatives 5j and 5k. Subsequent amide coupling with (15%) NH₃ in MeOH, EDC-HCl and HOBt in DMF afforded amides 5l and 5m in 82% and 85% yields, respectively. The synthesized compounds were evaluated against A-549, HeLa, DU-145, and MCF-7 cancer cell lines using the MTT assay. Experimental procedures, workup conditions, and yields are provided in the Experimental section.

3.2. Biological evaluation

The *in vitro* antiproliferative activity results for compounds 5a–5m against A-549, DU-145, HeLa, and MCF-7 cells are summarized in Table 2. Against A-549 cells, compounds 5b, 5d, 5l, and 5m showed the lowest IC₅₀ values in the synthesized series (4.14, 4.25, 5.82, and 4.36 μM, respectively). Against DU-145 cells, compounds 5b and 5d showed IC₅₀ values of 5.24 and 4.26 μM, respectively. Against HeLa cells, compounds 5b, 5d, and 5l showed IC₅₀ values of 3.16, 4.37, and 3.65 μM, respectively. Against MCF-7 cells, compounds 5b, 5d, 5l, and 5m showed IC₅₀ values of 4.29, 5.35, 4.16, and 4.85 μM, respectively. Overall, compounds 5b, 5d, 5l, and 5m showed the most favorable activity profiles across the tested cell-line panel, although all compounds were less potent than doxorubicin

under the reported conditions. Within the directly comparable 7- and 6-substituted pairs, the 7-methyl derivative 5c showed lower IC₅₀ values than the 6-methyl derivative 5f across the tested cell lines. Similar trends were observed for the 7-phenyl/6-phenyl pair 5d/5g and the 7-chloro/6-chloro pair 5b/5e. For example, compound 5d showed IC₅₀ values of 4.25–5.35 μM, whereas compound 5g showed values of 12.40–22.22 μM; compound 5b showed values of 3.16–5.24 μM, whereas compound 5e showed values of 8.46–16.42 μM. Among the phenyl-substituted derivatives, the tolyl compounds 5h and 5i showed IC₅₀ ranges of 7.34–14.23 and 11.35–16.43 μM, respectively; the benzoic acid derivatives 5j and 5k showed ranges of 8.12–22.15 and 11.34–18.23 μM, respectively; and the benzamide derivatives 5l and 5m showed ranges of 3.65–7.35 and 4.36–11.40 μM, respectively. These observations are limited to the tested compound set and cell lines.

4. Conclusion

A series of substituted 1-methyl-2-oxo-4-(1-(thiophene-2-carbonyl)azetidin-3-ylamino)-1, 2-dihydroquinoline-3-carbonitrile derivatives (5a–5m) was synthesized from substituted aminobenzoic acids through benzo[d][1,3]oxazine-2,4-dione intermediates using cyclization, displacement, chlorination, and coupling reactions. The synthesized compounds were evaluated against A-549, DU-145, HeLa, and MCF-7 cancer cell lines. Compounds 5b, 5d, 5l, and 5m were the most active members of the synthesized series, with IC₅₀ values of 3.16–11.40 μM. The matched 7- and 6-substituted pairs evaluated in this series showed lower IC₅₀ values for the 7-substituted analogues under the reported conditions. These findings represent preliminary *in vitro* screening results, and all synthesized compounds were less potent than doxorubicin.

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Disclosure statement

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Sample availability: Samples of the compounds are available from the corresponding author upon reasonable request.

CRedit authorship contribution statement

Conceptualization: Chandrakant Pawar, Harsh Gaikwad; Methodology: Chandrakant Pawar; Software: Lalaso Gaikwad, Sachin Kalme; Validation: Harsh Gaikwad, Lalaso Gaikwad, Sachin Kalme; Formal analysis: Harsh Gaikwad, Lalaso Gaikwad, Sachin Kalme; Investigation: Harsh Gaikwad, Lalaso Gaikwad, Sachin Kalme; Resources: Harsh Gaikwad and Chandrakant Pawar; Data Curation: Harsh Gaikwad, Lalaso Gaikwad, Sachin Kalme, Chandrakant Pawar; Writing—Original Draft: Harsh Gaikwad, Lalaso Gaikwad, Sachin Kalme; Writing—Review and editing: Chandrakant Pawar; Visualization: Harsh Gaikwad, Lalaso Gaikwad, Sachin Kalme; Funding acquisition: Chandrakant Pawar; Supervision: Chandrakant Pawar; Project Administration: Chandrakant Pawar.

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