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Synthesis of 4-methyl-*N*′-(3-alkyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazides with antioxidant, antitumor and antimicrobial activities†

Kodisundaram Paulrasu,[a] Arul Duraikannu,[b] Manikandan Palrasu,[c] Amirthaganesan Shanmugasundaram,[d] Murugavel Kuppusamy[e] and Balasankar Thirunavukkarasu*[f]

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The structures of the newly synthesized 4-methyl-*N*′-(3-alkyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (**5a–5l**) were confirmed by spectral and elemental analysis. The difference in the potency of activity against various free radicals, human cancer cells and microbial strains has been evaluated by SAR. Compounds with electron-donating methoxy (**5i** and **5c**) and methyl (**5h** and **5b**) substitutions at the *para* position of the phenyl showed excellent free radical scavenging effects. In the tested compounds, electron withdrawing fluoro (**5k** and **5e**), chloro (**5j** and **5d**), and bromo (**5l** and **5f**) substitution at the *para* position of the phenyl ring attached to C-2 and C-6 carbons of the piperidine moiety outperformed cytotoxic and antimicrobial activities. Our findings suggest that the antioxidant, anti-tumor and anti-microbial activities of compounds **5a–5l** create promising leads for the development of potent anti-tumor and anti-microbial agents.

Introduction

Reactive oxygen species (ROS) are essential for an organism's essential activities, such as the regulation of cell proliferation, intracellular signaling and synthesis of biologically active compounds and energy. However, excessive production of ROS causes oxidative stress and chronic diseases such as cardiovascular disease, diabetes, and cancer. ROS are known to directly interact with all types of biomolecules, including proteins, lipids, and DNA, resulting in oxidative cellular damage.[1–5] Oxidative stress has been implicated in all stages of the carcinogenic process.[1–6] A living organism has protective enzymatic and non-enzymatic antioxidant mechanisms against ROS-induced oxidative damage. Nevertheless, these protective systems are insufficient to prevent the damage entirely.[4–6] Furthermore, research over the past several decades has demonstrated that antioxidants play a protective role in multistage carcinogenesis.[4–6] Recently, considerable attention has been focused on identifying synthetic antioxidants that target various signaling pathways that are aberrant in cancer.

The piperidin-4-one nucleus, an important class of pharmacophore found in a wide variety of natural alkaloids, exhibits a wide spectrum of biological activities ranging from antibacterial to anticancer.[7,8] Many piperidine derivatives possess pharmacological activities including antimicrobial, antioxidant and anticancer activities and form an essential part of the molecular structure of important drugs.[7–11] Furthermore, modification of position 3 of the piperidin-4-one nucleus as well as a substitution of certain functional groups in the *para* position of the phenyl ring attached to C-2 and C-6 carbons of the piperidine moiety would result in compounds with potent biological activities. Therefore, many researchers have focused on modifying the piperidin-4-one pharmacophore to achieve better biological activities.[7–9] Hydrazones contain an azomethine –NHN=CH– proton that constitutes an important class of compounds for new drug development. Hydrazide–hydrazone derivatives received the attention of various medic-

[a]*Department of Chemistry, Annamalai University, Annamalainagar, 608002, Tamilnadu, India*
[b]*Department of Clinical Neurosciences, University of Calgary, Calgary, Alberta T2N 4N1, Canada*
[c]*Department of Pharmacology, University of Maryland School of Medicine, Baltimore, Maryland 21201*
[d]*Department of Chemistry, Saveetha School of Engineering, Saveetha University, Thandalam, Chennai-602 105, Tamilnadu, India*
[e]*Department of Chemistry, Saveetha Engineering College, Thandalam, Chennai-602 105, Tamilnadu, India*
[f]*Chemistry Section, FEAT, Annamalai University, Annamalainagar, 608002, Tamilnadu, India. E-mail: drtbalasankar@rediff.com; Fax: +91 4144 239141; Tel: +91 9944472276*

†Electronic supplementary information (ESI) available: Spectral data (^{1}H NMR, ^{13}C NMR, H–H COSY, NOESY, HSQC, and HMBC) for compounds **5a** and **5g**. See DOI: 10.1039/c4ob00739e

inal chemists as a result of their effectual biological potencies, *viz.*, antimicrobial, anti-tubercular, and also anticonvulsant activities.[8,10,11] Recently, we have documented a synthesis system that combines 3-azabicyclonone and thiadiazole moieties together to produce the corresponding hydrazones with promising antimicrobial activities.[12] Similarly, the present study aims to prove that the addition of hydrazide into the piperidin-4-one pharmacophore with different modifications would result in compounds with potent biological activities.

In the present study, a new series of 4-methyl-*N*′-(3-methyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide **5(a–f)** and 4-methyl-*N*′-(3-ethyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide **5(g–l)** was synthesized using a reaction of piperidin-4-one with 4-methyl-1,2,3-thiadiazole-5-carboxylic acid hydrazide in the presence of acetic acid in methanol. The chemical structures were confirmed using IR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis. In addition, we executed structure–activity relationship studies using newly synthesized hydrazone derivatives with potent antioxidant, anti-tumour and antimicrobial activities.

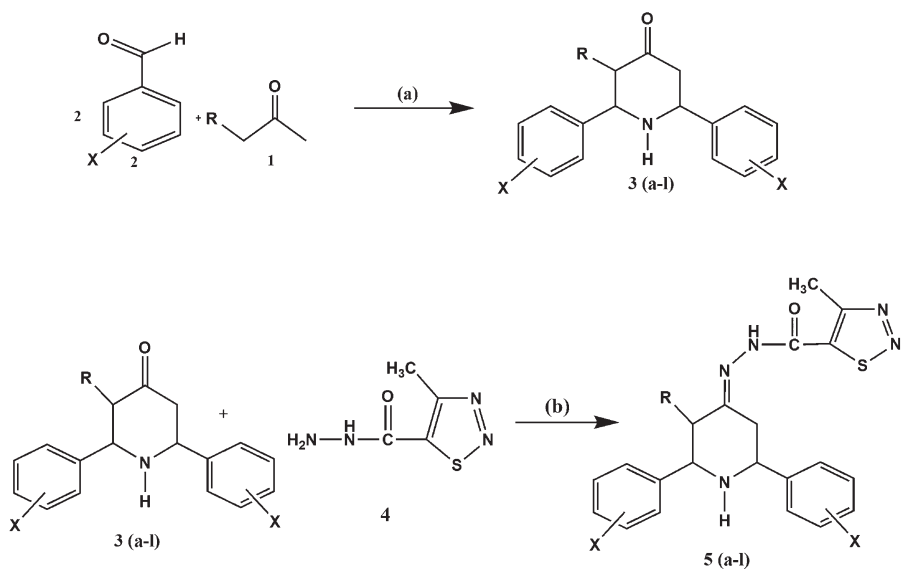
Results and discussion

Chemistry

Syntheses of 4-methyl-*N*′-(3-methyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide **5(a–f)** and 4-methyl-*N*′-(3-ethyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide **5(g–l)** were carried out according to the steps shown in Fig. 1. A detailed investigation of IR, ^{1}H NMR and C^{13} NMR spectral data with CHN analysis (Table 1) was performed to identify and establish the newly-synthesized compounds (**5a–l**). For **5a**, all ^{1}H and ^{13}C signals have been assigned unambiguously using ^{1}H–^{1}H-COSY, NOESY, HSQC and HMBC spectra.

Structural elucidation of compound 5a

In ^{1}H NMR spectra for compound **5a**, a broad and more downfield D_2O exchangeable singlet at 10.80 ppm was characteristic of the NH amide group. Another, broad singlet signal resonated at 2.11 ppm was assigned for the NH proton of the piperidin-4-one ring. Signal broadening is due to the faster exchange of the NH proton with solvent moisture than the resonance time scale. Two



Reagents and conditions : (a) CH_3COONH_4, C_2H_5OH, 70 °C
(b) CH_3OH, CH_3COOH, 2h

Sample code	R	X
3a, 5a	$-CH_3$	H
3b, 5b	$-CH_3$	p-CH_3
3c, 5c	$-CH_3$	p-OCH_3
3d, 5d	$-CH_3$	p-Cl
3e, 5e	$-CH_3$	p-F
3f, 5f	$-CH_3$	p-Br
3g, 5g	$-CH_2CH_3$	H
3h, 5h	$-CH_2CH_3$	p-CH_3
3i, 5i	$-CH_2CH_3$	p-OCH_3
3j, 5j	$-CH_2CH_3$	p-Cl
3k, 5k	$-CH_2CH_3$	p-F
3l, 5l	$-CH_2CH_3$	p-Br

Fig. 1 Schematic representation of the synthesis of 4-methyl-*N*′-(3-methyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide **5(a–f)** and 4-methyl-*N*′-(3-ethyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazides **5(g–l)**.

Table 1 Analytical data of compounds **5a–5l**

Compounds	Molecular formula	Molecular weight	Elemental analysis found[a] (calculated) (%)		
			C	H	N
5a	$C_{22}H_{23}N_5OS$	405.52	65.06 (65.16)	5.62 (5.72)	17.21 (17.27)
5b	$C_{24}H_{27}N_5OS$	433.57	66.42 (66.48)	6.26 (6.28)	16.11 (16.15)
5c	$C_{24}H_{27}N_5O_3S$	465.57	61.89 (61.92)	5.75 (5.85)	15.01 (15.04)
5d	$C_{22}H_{21}Cl_2N_5OS$	474.41	55.69 (55.70)	4.41 (4.46)	14.73 (14.76)
5e	$C_{22}H_{21}F_2N_5OS$	441.50	59.03 (59.85)	4.74 (4.79)	15.81 (15.86)
5f	$C_{22}H_{21}Br_2N_5OS$	563.31	46.89 (46.91)	3.70 (3.76)	12.40 (12.43)
5g	$C_{23}H_{25}N_5OS$	419.54	65.80 (65.84)	6.00 (6.01)	16.63 (16.69)
5h	$C_{25}H_{29}N_5OS$	447.60	67.02 (67.08)	6.49 (6.53)	15.62 (15.65)
5i	$C_{25}H_{29}N_5O_3S$	479.59	62.59 (62.61)	6.01 (6.09)	14.58 (14.60)
5j	$C_{23}H_{23}Cl_2N_5OS$	487.10	56.49 (56.56)	4.70 (4.75)	14.29 (14.34)
5k	$C_{23}H_{23}F_2N_5OS$	453.16	60.59 (60.64)	5.03 (5.09)	15.33 (15.37)
5l	$C_{23}H_{23}Br_2N_5OS$	577.33	47.81 (47.85)	4.00 (4.02)	12.09 (12.13)

[a] The observed microanalysis values for C, H and N were within ±0.4% of the theoretical values.

doublets were observed in the region of 1.17 and 3.63 ppm due to H-2a and the methyl group of C-3 at the piperidin ring. Three doublets were observed in the region of 3.43, 2.44 and 3.95 ppm due to H-5e, H-5a and H-6a. A broad singlet signal appeared at 2.53 ppm, corresponding to three proton integrals attributed to the methyl group of the thiadiazole ring. A multiple signal appeared at 2.76 ppm corresponding to one proton integral due to H-3a.

In ^{13}C NMR of compound **5a**, two downfield resonances at 162.2 and 159.8 ppm were assigned to C=N (C-9) and C=O (=N–NH–*CO*–) carbons, respectively. The carbon resonances observed around 142.1 and 142.7 ppm were due to *ipso* carbons. However, there were four signals around 69.35, 46.3, 37.1 and 61.2 ppm, which were conveniently assigned to the C-2, C-3, C-5 and C-6 carbons, respectively. The ^{13}C chemical shift values of the two methyl carbons (C-3 at the piperidin ring and C-4 at the thiadiazole ring) were observed at 13.9 and 14.8 ppm. The signals at 164.5 and 135.0 ppm were assigned to C-4 and C-5 of the thiadiazole ring. Taken together, all the above observations substantiate the proposed structure of some 4-methyl-*N*′-(3-alkyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazides (**5a–5l**).

Biological activity

Piperidin-4-one pharmacophores are found to possess a wide range of pharmacological properties. Molecules containing azomethine –NHN=CH– groups constitute an important class of compounds for new drug development.[7,8,10] Therefore, we have developed the system that combines piperidin-4-one pharmacophore and hydrazide moieties together to produce the corresponding hydrazones (**5a–5l**) with the anticipation of several promising antioxidant, anticancer and antimicrobial agents emerging. The present study also aimed to investigate the structure–activity relationship for the antioxidant, cytotoxic and antimicrobial activities of hybrid molecules containing the piperidin-4-one pharmacophore and hydrazones.

In vitro free radical scavenging effects

Twelve different hydrazone derivatives were synthesized and evaluated for their *in vitro* free radical scavenging activity against various free radicals. Our findings provide evidence that synthetic compounds (**5a–5l**) showed a concentration-dependent anti-radical activity resulting from reduction of DPPH$^{\bullet}$, ABTS$^{\bullet+}$, O$^{\bullet-}$, OH$^{\bullet}$, and nitric oxide radicals to their non-radical forms. IC_{50} values for the free radical scavenging effects of ascorbic acid and various synthetic compounds (**5a–5l**) are shown in Table 2.

It is well known that an increase in antioxidant activity is observed with replacement of alkyl chains such as methyl, ethyl to phenyl rings due to the electron resonance effect of the phenyl group.[13] The results of the present study demonstrate the presence of an ethyl group substitution in position 3 of piperidin-4-one compounds (**5g–5l**) exerts greater inhibitory effects against various free radicals compared to the compounds substituted with methyl groups in position 3 of piperidin-4-one (**5a–5f**). Several studies have demonstrated that organic molecules incorporating an electron donating group (amine, hydroxyl, methoxy and alkyl) at the *para* position of the phenyl ring can act as free radical trapping agents and are capable of opposing oxidative challenges.[7,14–16] Compounds possessing electron-donating methoxy (**5i**) and methyl (**5h**) substitutions at the *para* position of the phenyl ring attached to the C-2 and C-6 carbons of the piperidine moiety showed excellent free radical scavenging effects compared to standard antioxidant ascorbic acid, a known antioxidant used as a positive control. Compounds with electron-donating methoxy (**5c**) and methyl (**5b**) substitutions at the *para* position of the phenyl ring attached to the C-2 and C-6 carbons and methyl substitution at position 3 of piperidin-4-one compounds showed remarkable activities. These findings confirm reports by other workers on the *in vitro* free radical scavenging effects of organic molecules incorporating an electron donating group (amine, hydroxyl, methoxy and alkyl) at the *para* position of the phenyl ring.[14–16]

Compounds **5a** and **5g** deprived of any substitutes at the *para* position of the phenyl groups at the C-2 and C-6 positions of the piperidine ring showed moderate *in vitro* free radical scavenging effects against various free radicals. Compounds possessing electron-withdrawing bromo (**5l** and **5f**), chloro (**5j**

Table 2 IC_{50} values for free radical scavenging activity and MTT assay[a]

Compound	IC_{50} values for free radical scavenging activity (μg ml^{-1})					IC_{50} values for MTT assay (μmol)		
	DPPH	ABTS	Superoxide	Hydroxyl	Nitric oxide	HeLa	A549	NL20
Ascorbic acid	4.23	4.52	4.19	5.53	6.32	—	—	—
5a	5.85	5.53	4.36	6.53	8.59	7.91	11.28	12.35
5b	4.72	4.76	3.88	5.62	7.09	11.25	10.88	11.89
5c	3.90	4.12	3.69	5.00	5.92	4.27	6.30	7.56
5d	14.96	14.96	16.83	14.93	17.82	2.28	3.40	4.63
5e	11.82	11.82	12.12	14.89	16.23	1.41	2.61	12.36
5f	8.38	8.38	8.01	10.28	12.86	3.91	5.41	8.12
5g	5.53	4.92	4.13	5.98	7.93	10.20	13.71	12.45
5h	3.53	3.87	3.02	4.59	6.12	13.34	15.30	18.56
5i	3.03	3.32	2.21	4.20	5.13	4.94	8.44	9.23
5j	9.53	10.53	10.58	10.91	13.8	3.10	4.57	5.90
5k	12.87	11.38	14.32	14.82	13.40	2.51	3.60	11.12
5l	7.95	7.18	7.02	10.51	11.08	4.21	6.07	7.61

[a] IC_{50} values were determined by plotting dose–response curves of radical scavenging activities *vs.* the concentration of synthetic compounds using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., San Diego, CA).

and **5d**), and fluoro (**5k** and **5e**) substitutions at the *para* position of the piperidine moiety showed admirable *in vitro* free radical scavenging effects against various free radicals. These admirable or less free radical scavenging effects of compounds with bromo, chloro and fluoro substitutions may be due to the electron-withdrawing inductive effect of halogens. Our results are in line with other findings.[13,16,17] Research over the past several decades has demonstrated that excessive production of toxic radical species is known to cause deleterious changes in DNA, lipid, and protein oxidation. Thus, free radicals may serve as a source of mutations that initiate carcinogenesis.[4,5] Our results provide evidence that the ability of synthetic compounds to quench $O^{\bullet -}$, $OH^{\bullet}$, and nitric oxide radicals is directly associated with the prevention of oxidative stress and free radical-induced carcinogenesis.

Anticancer effects

We investigated the cytotoxic effects of newly synthesized hydrazone derivatives with hydrazide and various substituents on piperidone-4-one pharmacophore (**5a–5l**) on human lung epithelial carcinoma A549 and HeLa cell growth using the MTT assay in order to validate their anticancer effects and to correlate their antioxidant activity. Consistent with free radical scavenging activity, all the synthesized compounds significantly inhibited the proliferation of cancer cells in a dose-dependent manner (0, 3, 6, 9, 12, 15, and 18 μM) after 24 h of incubation. The inhibitory effects of synthetic compounds were in the order: **5e** > **5k** > **5d** > **5j** > **5f** > **5l** > **5c** > **5i** > **5a** > **5g** > **5b** > **5h**. IC_{50} values for the cytotoxic effects of various synthetic compounds (**5a–5l**) are shown in Table 2. Generally compounds containing electron withdrawing functional groups (–F, –Cl) exhibited more potent cytotoxic effects against the tested cancer cells compared to the electron donating functional groups ($–CH_3$, $–OCH_3$) present on the aryl rings attached to piperidones. Our results are in line with those of other workers.[7,8,18–20]

Contrary to reports of a positive correlation between the cytotoxicity and the antioxidant capacity of natural and synthetic compounds,[21] we found low *in vitro* antioxidant activity in synthetic compounds **5e** and **5k** despite their high cytotoxicity. The strong cytotoxicity with poor antioxidant properties of synthetic compounds **5e** and **5k** may be due to the pro-oxidant effects by the electron withdrawing halogens. Although potent antioxidants often possess strong pro-oxidant activity, we found low cytotoxicity in synthetic compounds with electron donating functional groups ($–CH_3$, $–OCH_3$) present on the aryl rings attached to piperidones in line with the observations of Lee *et al.*[22] However, multiple mechanisms regulating the antioxidant and cytotoxic effects of the hybrid molecules need to be further investigated.

We also compared the cytotoxicity of the hydrazone derivatives in A549 lung cancer cells and NL-20 normal lung epithelial cells. Most of the compounds displayed significant cytotoxic effects in A549 cells, although to different extents; the synthetic compounds containing electron withdrawing functional groups (–F, –Cl) exhibited more potent cytotoxic effects against lung cancer cells. However, these compounds displayed less cytotoxicity to NL-20 normal lung epithelial cells. In particular, the results of compounds **5e** and **5k** seem to suggest a strikingly differential effect on cancer cells. Other compounds, which were toxic to lung cancer cells, were toxic to normal lung cells to more or less the same extent as to cancer cells. Nevertheless, the mechanisms responsible for the differential cytotoxic effects of **5e** and **5k** remain unclear and require further investigation.

Of the several synthetic compounds, only **5e** and **5k**, which were demonstrated to exert potent cytotoxic effects from each series, were used for testing their antiproliferative effects by crystal violet blue staining assay in comparison with MMC (50 ng) (Fig. 2). We have treated the A549 cells with synthetic compounds **5e** and **5k** at 2.5, 5, and 10 μM. Compounds **5e** and **5k** at 5 and 10 μM showed a greater inhibitory effect on

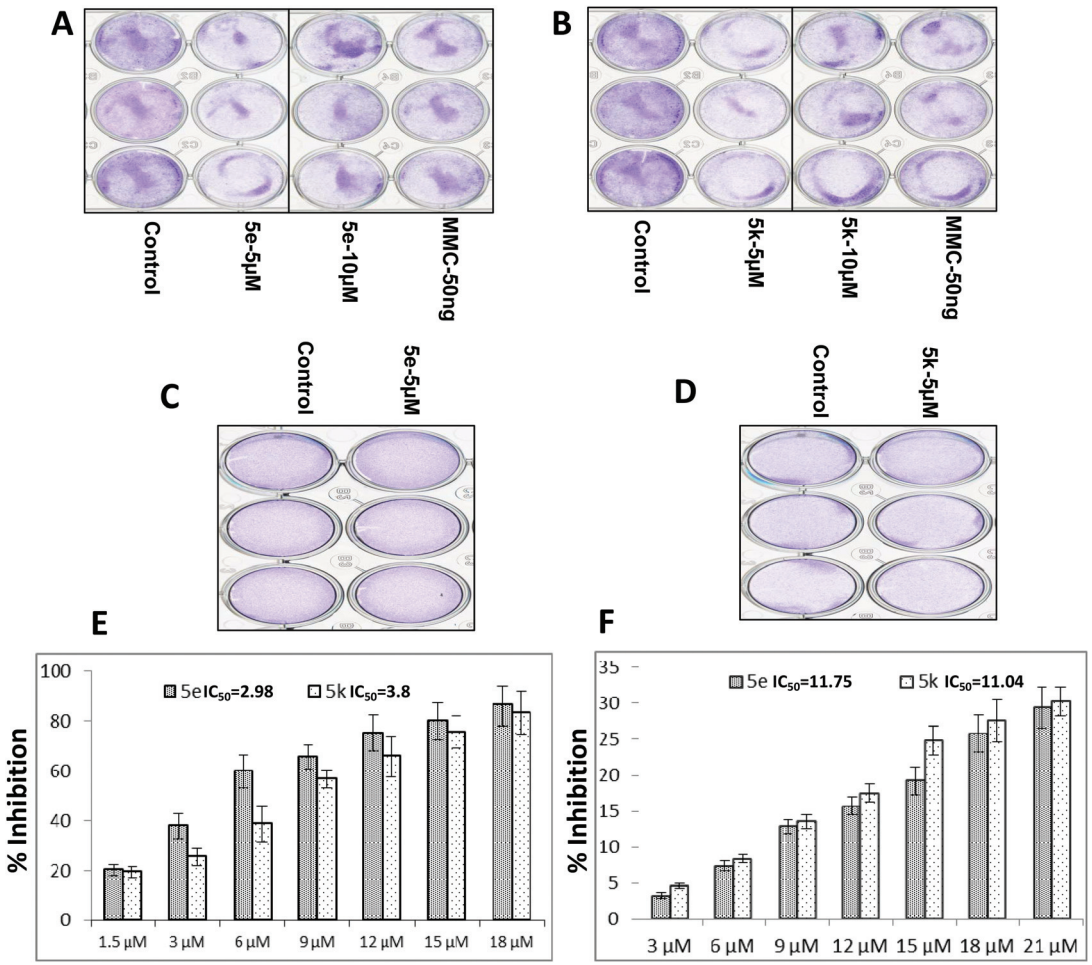


Fig. 2 Antiproliferative effects of synthetic compounds **5e** and **5k** in A549 (A, B and E) and NL 20 (C, D and F) cells by the crystal blue staining method. A549 cells and NL 20 cells were plated at day 0 with an equal number. After overnight incubation, A549 and NL 20 cells were treated with compounds **5e** (A and C) and **5k** (B and D) for 24 h. The representative images on day 1 were shown for cells growing in medium containing synthetic compounds **5e** (A) and **5k** (5B) or MMC. IC_{50} values for the synthetic compounds **5e** and **5k** in A549 (E) and NL20 (F) cells were measured using the crystal violet blue staining method.

A549 cells and the medium dose of 5 μM was more effective when compared to other doses and MMC, the standard anticancer agent. IC_{50} values for the synthetic compounds **5e** and **5k** using the crystal blue staining assay are shown in Fig. 2E and 2F. The crystal violet blue staining assay using compounds **5e** and **5k** also strongly supports its antiproliferative effects against A549 cells. The cytotoxicity of **5e** and **5k** was also tested in normal lung epithelial cells (NL-20). Encouragingly, **5e** and **5k** have less cytotoxic effects on cell viability in NL-20 cells as compared to lung cancer cell lines A549. Both compounds showed only approximately 10% reduction in cell viability in the NL-20 cells.

Furthermore, the anti-invasive potential of synthetic compounds was examined by cell a migration assay. Control cells have a strong invasive potential as revealed by the increased number of cells (Fig. 3A and D). However, HeLa cells treated with 5 μM **5e** and **5k** can mitigate the invasive potential of cells (Fig. 3B, C and D). Although a methyl substitution at position 3 of piperidin-4-one compound **5e** exerts greater inhibitory effects against HeLa and A549 cells in MTT assays, no significant difference was observed between a methyl (**5e**) and ethyl (**5k**) substitution at position 3 of piperidin-4-one by crystal violet blue staining and cell migration assays. These results demonstrate that the synthetic compounds containing electron withdrawing fluoro functional groups can inhibit the growth and invasive potential of cancer cells and act as potent anticancer agents.

Antibacterial and antifungal activity

In vitro antibacterial activity of the synthesized hydrazones was carried out against *B. subtilis*, *S. aureus, K. pneumoniae P. aeruginosa* and *E. coli* by the twofold serial dilution method using Streptomycin as the standard. The MIC values are presented in Table 3. Analysis of *in vitro* antimicrobial effects of all the 4-methyl-*N*′-(3-alkyl-2,6-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazides (**5a–5l**) revealed a diverse range of inhibitory activity (6.25–200 μg ml^{-1}) against all the pathogens except compounds **5a** and **5h**, which did not show activity

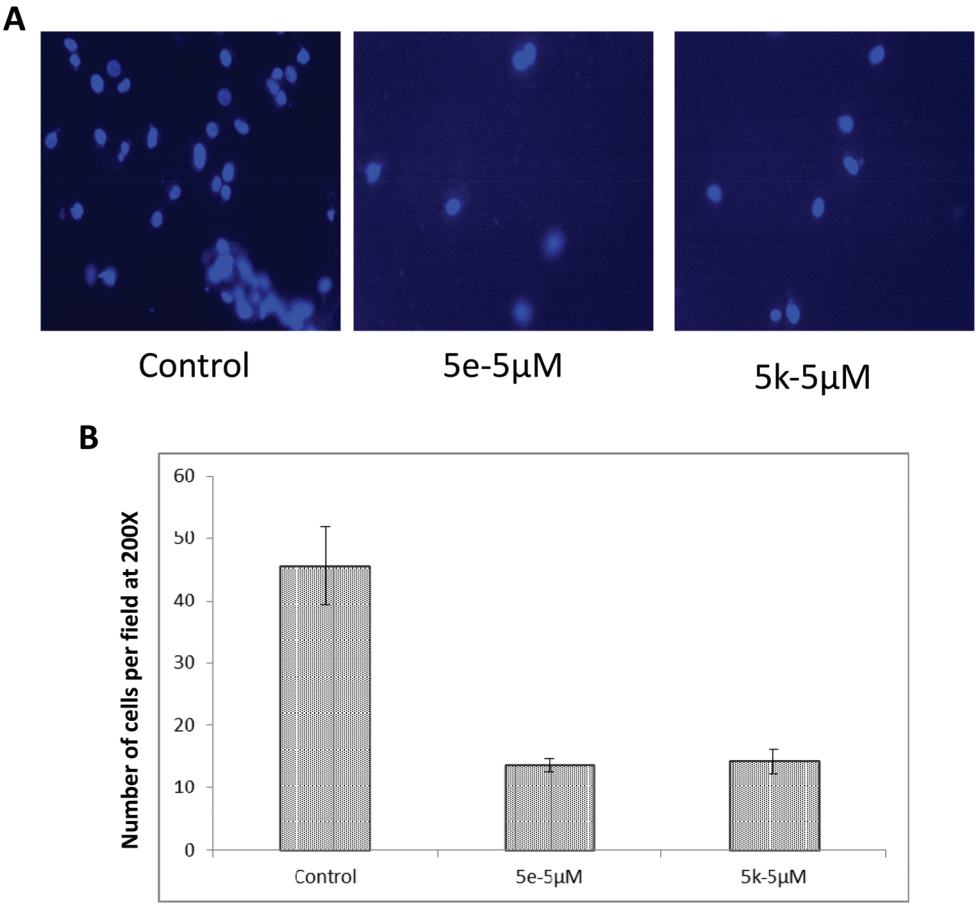


Fig. 3 Anti-invasive potential of synthetic compounds **5e** and **5k** by cell migration assay. Synthetic compounds **5e** and **5k** block the invasive advantage of HeLa cells. HeLa cells were grown in 5 μM **5e** and **5k** overnight and plated into transwells the following day in medium containing 5 μM **5e** and **5k**. Transwells were processed accordingly 2 hours after plating. Cells were counted with 10 random fields at a magnification of 200×. Representative images of the invasive cell density are shown in Fig. 2A. All counted cell numbers were used to plot the relative invasive potential (B). Control cells have a stronger invasive potential. HeLa cells treated with 5 μM **5e** and **5k** can mitigate the invasive potential of cells.

Table 3 *In vitro* antibacterial (MIC μM ml^{-1}) of compounds **5a–5l** by the 2-fold serial dilution method

	MIC (μM ml^{-1})				
Compounds	*B. subtilis*	*S. aureus*	*K. pneumoniae*	*S. aeruginosa*	*E. coli*
5a	50	200	200	100	100
5b	100	50	100	200	200
5c	25	25	50	50	100
5d	12.5	12.5	25	25	25
5e	**6.25**	**3.12**	**3.12**	**6.25**	**6.25**
5f	12.5	25	25	50	50
5g	50	200	200	50	100
5h	200	100	>200	100	>200
5i	100	50	50	100	100
5j	12.5	25	50	25	50
5k	**25**	**6.25**	**12.5**	**12.5**	**12.5**
5l	12.5	25	25	50	50
Streptomycin	25	12.5	25	12.5	12.5

MIC, minimum inhibitory concentration.

against *K. pneumoniae* and *P. Aureus*, even at a maximum concentration of 200 μg ml^{-1}. The compounds deprived of any substitutes at the *para* position of the phenyl groups at the C-2 and C-6 positions of the heterocyclic ring (**5a** and **5g**) hinder the growth of *Bacillus subtilis, S. aureus* and *E. coli* at a MIC value of 50–100 μg ml^{-1}. However, compounds possessing *para* fluoro (**5e** and **5k**) at aryl groups in the piperidine moiety accounts for the enhanced inhibitory effects against *B. subtilis, P. Aeruginosa*, *K. pneumoniae, S. aureus and E. coli* at MIC values of 3.13–25 μg ml^{-1} when compared to the standard antibiotic streptomycin. Substitution of electron withdrawing chloro (**5d**/**5j**), and bromo (**5f**/**5l**) functional groups at aryl groups showed modest antibacterial activity against *B. subtilis, P. aeruginosa*, *K. pneumoniae, S. aureus and E. coli* at MIC values of 12.5–50 μg ml^{-1} similar to that of standard streptomycin. Several studies have documented that electron withdrawing substitutes like fluoro, chloro and bromo substituted 2,6-diaryl piperidone derivatives exerted excellent antibacterial and antifungal activities.[7,8,10–12,14,23] Compounds **5b**/**5h** and **5c**/**5i** possessing electron donating methyl and methoxy substitutions, respectively, at the *para* position of phenyl rings

Table 4 *In vitro* antifungal (MIC μM ml^{-1}) of compounds **5a–5l** by the 2-fold serial dilution method

	MIC (μM ml^{-1})			
Compounds	*C. albicans*	*Rhizopus* sp.	*A. niger*	*A. flavus*
5a	50	>200	100	100
5b	50	100	200	>200
5c	50	100	25	25
5d	50	12.5	25	6.25
5e	**6.25**	**3.12**	**6.25**	**6.25**
5f	25	12.5	12.5	25
5g	50	>200	100	100
5h	150	200	150	100
5i	50	25	100	50
5j	25	50	12.5	25
5k	**12.5**	**6.25**	**12.5**	**12.5**
5l	25	25	50	12.5
Fluconazole	25	12.5	6.25	25

MIC, minimum inhibitory concentration.

attached to C-2 and C-6 carbons of the piperidine moiety show moderate antibacterial activity against all the tested bacterial strains in the range of 25–200 μg ml^{-1}.

The results of the present study also provide evidence for the antifungal effects of an array of 4-methyl-*N*′-(3-alkyl-2,6-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazides (**5a–5l**) with the MIC value ranging from 6.25 to 100 μg ml^{-1} (Table 4). Compounds **5a** and **5g** that lack any substitutes at the aryl groups showed mild and comparatively less antifungal activity against *A. flavus* (100 μg ml^{-1}) and *C. albicans* (50 μg ml^{-1}) compared to fluconazole, a known antifungal agent used as a positive control.

Compounds possessing electron withdrawing fluoro substituents at aryl groups (**5e**/**5k**) exerted four/two fold increase in antifungal activity against *C. albicans, Rhizopus and A. flavus* at MIC values of 6.25–12.5 μg ml^{-1} when compared to the standard fluconazole. However, compound **5e**/**5k** showed inhibitory activity against *A. niger* similar to that of standard fluconazole. Substitution of electron withdrawing chloro (**5d**/**5j**) and bromo (**5f**/**5l**) functional groups at aryl groups exerted modest antifungal activity against *C. albicans*, *Rhizopus*, *A. flavus* and *A. niger* at MIC values of 6.25–50 μg ml^{-1}. Furthermore, a phenyl ring with electron-donating methoxy and methyl groups at the *para* position ((**5c**/**5i**) and (**5b**/**5h**)) hinder the growth of all fungal strains at MIC values ranging from 25–100 μg ml^{-1}.

Conclusions

Combining piperidin-4-one pharmacophore with hydrazide moieties is gaining increasing attention as a promising strategy for the development new hydrazine derivatives (**5a–5l**) with potent biological activities. Synthesized compounds (**5a–5l**) are examined for their antioxidant, anticancer and antimicrobial activities. Although the synthetic compounds with electron-donating methoxy (**5i**) and methyl (**5h**) substitutions at the *para* position of the phenyl ring attached to the C-2 and C-6 carbons of the piperidine moiety were more effective than the compounds **5k** and **5e** in terms of *in vitro* antioxidant activity, the synthetic compounds **5k** and **5e** also possessed significant antioxidant potential. The strong *in vitro* free radical scavenging effects of organic molecules (**5i**, **5h**, **5c**, and **5b**) may be due to the presence of electron donating groups (methoxy and alkyl) at the *para* position of the phenyl ring. However, compounds possessing electron-withdrawing bromo (**5l** and **5f**), chloro (**5j** and **5d**), and fluoro (**5k** and **5e**), substitutions at the *para* position of the two phenyl rings of the piperidine moiety exerted a wide range of free radical scavenging activities and showed more cytotoxicity to cancer cells and antimicrobial activities. This may be due to the presence of azomethine –NHN═CH– groups as well as electron withdrawing groups at the *para* position of the aromatic ring of the piperidine moiety. In contrast to free radical scavenging effects, the presence of a methyl group substitution in position 3 of the piperidin-4-one compounds (**5a–5f**) exerts greater inhibitory effects against various cancer cells and microbes compared to the compounds substituted with ethyl groups in position 3 of piperidin-4-one (**5g–5l**). A noteworthy consideration is the fact that the synthetic compounds **5k** and **5e** selectively exert their cytotoxicity in cancer cells, but not in normal cells. Among the compounds tested, **5e** and **5k** were selected as the lead compounds for further studies. Studies on the molecular mechanisms by which the synthetic compound exerts its antitumor and antimicrobial activities are making headway and will be reported on in the future.

Materials and methods

Materials and apparatus

All chemicals that were purchased were used without further purification. All the reported melting points that were measured in open capillaries were uncorrected. FT-IR analysis was done by making a pellet of compound with KBr. Both one and two dimensional NMR spectra were recorded in the NMR spectrometer. A sample was prepared with a 5 mm diameter tube using DMSO-d_6 solvent (10 mg in 0.5 ml). ^{1}H NMR and ^{13}C NMR data were collected in 400.13 MHz and 100.62 MHz operating frequency, respectively. Chemical shifts (δ) were expressed in ppm with respect to TMS. Splitting patterns were designated as follows: s-singlet, d-doublet, t-triplet, q-quartet and m-multiplet.

General procedure for the synthesis of 4-methyl-*N*′-(3-alkyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazides 5(a–l)

The 3-alkyl-2r,6c-diaryl piperidin-4-one **3(a–l)** were prepared by the condensation of appropriate ketones, aldehydes and ammonium acetate in a 1 : 2 : 1 ratio, according to the method described by Noller and Baliah.[24] A reaction mixture containing 3-alkyl-2r,6c-diaryl piperidin-4-one **3(a–l)** (1 mmol) and 4-methyl-1,2,3-thiadiazole-5-carboxylic acid hydrazide (1.5 mmol) was dissolved in the solvent mixture of chloroform and methanol (1 : 1 v/v) and acetic acid (2 ml) was added as a catalyst. The reaction mixture was refluxed for about 3–4 hours. After

completion of the reaction, the crude product was formed, filtered and washed with a cold mixture of ethanol and water. The pure compounds **5** (**a–l**) were obtained by crystallization from distilled ethanol. Analytical data of compounds **5a–5l** are shown in Table 1.

4-Methyl-*N*′-(3-methyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5a). White solid, yield: 70%, m.p: 177 °C. IR (KBr, ν_{max} cm^{-1}): 1653 (C═N). ^{1}H NMR (δ, 400 MHz-$CDCl_3$, ppm): 1.17 (d, 3H, CH_3 at piperidin ring), 2.11 (s, 1H, NH at piperidin ring), 2.44 (dd, 1H, C5-1Ha), 2.53 (s, 3H, CH_3 at thiadiazole ring), 2.76 (m, 1H, C3-1H), 3.44 (dd, 1H, C5-1He), 3.64 (d, 1H, C2-1H), 3.96 (dd, 1H, C6-1H), 7.28–7.51 (m, 10H, Ar–H), 10.80 (s, 1H, N–H, amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 13.9 (CH_3 at piperidin ring), 14.8 (CH_3 at thiadiazole ring), 37.1 (C-5), 46.3 (C-3), 61.2 (C-6), 69.3 (C-2), 126.7–128.8 (Ar–C), 135.0 (C-5 at thiadiazole ring), 142.1 and 142.7 (*ipso* carbons), 159.8 (C-4), 162.2 (NHCO) and 164.5 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-methyl-2r,6c-bis(*p*-methylphenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5b). White solid, yield: 65%, m.p: 170 °C, IR (KBr, ν_{max} cm^{-1}): 1647 (C═N). ^{1}H NMR (δ, 400 MHz-$CDCl_3$, ppm): 1.15 (d, 3H, CH_3 at piperidin ring), 2.07 (s, 1H, NH at piperidin ring), 2.37 (dd, 1H, C5-1Ha), 2.38 (s, 6H, CH_3 at phenyl ring), 2.75 (s, 3H, CH_3 at thiadiazole ring), 2.75 (m, 1H, C3-1H), 3.21 (d, 1H, C5-1He), 3.56 (d, 1H, C2-1H), 3.87 (d, 1H, C6-1H), 7.15–7.36 (m, 8H, Ar–H), 9.96 (s, 1H, N–H, amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 13.8 (CH_3 at piperidin ring), 15.0 (CH_3 at thiadiazole ring), 21.1 (CH_3 at phenyl ring), 36.9 (C-5), 46.2 (C-3), 60.8 (C-6), 69.0 (C-2), 126.5–129.3 (Ar–C), 135.1 (C-5 at thiadiazole ring), 137.7 and 139.7 (*ipso* carbons), 159.5 (C-4), 161.5 (NHCO), and 164.5 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-methyl-2r,6c-bis(*p*-methoxyphenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5c). White solid, yield: 73%, m.p: 160 °C. IR (KBr, ν_{max} cm^{-1}): 1651 (C═N). ^{1}H-NMR (δ, 400 MHz-$CDCl_3$, ppm): 1.14 (d, 3H, CH_3 at piperidine ring), 2.04 (s, 1H, NH at piperidin ring), 2.37 (t, 1H, C5-1Ha), 2.69 (s, 1H, CH_3 at thiadiazole ring), 2.69 (m, 1H, C3-1H), 3.25 (dd, 1H, C5-1He), 3.54 (d, 1H, C2-1H), 3.80 (dd, 1H, C6-1H), 3.88 (s, 6H, OCH_3), 6.86–7.41 (m, 8H, Ar–H), 10.32 (s, 1H, N–H amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 13.8 (CH_3 at piperidin ring), 15.0 (CH_3 at thiadiazole ring), 37.1 (C-5), 46.4 (C-3), 55.3 (OCH_3), 60.5 (C-6), 68.7 (C-2), 113.8–128.8 (Ar–C), 134.3 and 134.9 (*ipso* carbons), 135.1 (C-5 at thiadiazole ring), 159.4 (C-4), 161.8 (NHCO) and 164.5 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-methyl-2r,6c-bis(*p*-chlorophenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5d). White solid, yield: 70%, m.p: 204 °C. IR (KBr, ν_{max} cm^{-1}): 1651 (C═N). ^{1}H NMR (δ, 400 MHz-$CDCl_3$, ppm): 1.14 (s, 3H, CH_3 at piperidin ring), 2.08 (s, 1H, NH at piperidin ring), 2.36 (dd, 1H, C5-1Ha), 2.64 (s, 3H, CH_3 thiadiazole ring), 2.69 (m, 1H, C3-1H), 3.35 (dd, 1H, C5-1He), 3.62 (d, 1H, C2-1H), 3.93 (dd, 1H, C6-1H), 7.32–7.44 (m, 8H, Ar–H), 10.51 (s, 1H, N–H, amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 13.7 (CH3 at piperidin ring), 14.9 (CH_3 at thiadiazole ring), 36.9 (C-5), 46.2 (C-3), 60.4 (C-6), 68.4 (C-2), 128.0–129.1 (Ar–C), 134.9 (C-5 at thiadiazole ring), 140.3 and 141.0 (*ipso* carbons), 158.7 (C-4), 162.0 (NHCO) and 164.4 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-methyl-2r,6c-bis(*p*-fluorophenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5e). White solid, yield: 78%, m.p: 188 °C. IR (KBr, ν_{max} cm^{-1}): 1651 (C═N). ^{1}H NMR (δ, 400 MHz-$CDCl_3$, ppm): 1.13 (d, 3H, CH_3 at piperidin ring), 2.09 (s, 1H, NH at piperidin ring), 2.73 (m, 1H, C3-1H), 2.41 (dd, 1H, C5-1Ha), 2.49 (s, 3H, CH_3 at thiadiazole ring), 3.42 (dd, 1H, C5-1He), 3.61 (d, 1H, C2-1H), 3.92 (dd, 1H, C6-1H), 7.25–7.48 (m, 8H, Ar–H), 10.78 (s, 1H, N–H, amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 13.8 (CH_3 at piperidin ring), 14.7 (CH_3 at thiadiazole ring), 37.1 (C-5), 46.3 (C-3), 61.2 (C-6), 69.3 (C-2), 126.6–128.7 (Ar–C), 134.9 (C-5 at thiadiazole ring), 142.0 and 142.6 (*ipso* carbons), 159.8 (C-4), 162.20 (NH CO) and 164.4 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-methyl-2r,6c-bis(*p*-bromophenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5f). White solid, yield: 65%, m.p: 220 °C. IR (KBr, ν_{max} cm^{-1}): 1629 (C═N). ^{1}H NMR (δ, 400 MHz-$CDCl_3$, ppm): 1.13 (d, 3H, CH_3 at piperidin ring), 2.17 (s, 1H, NH at piperidin ring), 2.39 (dd, 1H, C5-1Ha), 2.68 (s, 3H, CH_3 at thiadiazole ring), 2.73 (m, 1H, C3-1H), 3.27 (dd, 1H, C5-1He), 3.59 (d, 1H, C2-1H), 3.93 (d, 1H, C6-1H), 7.26–7.49 (m, 8H, Ar–H), 10.13 (s, 1H, N–H, amide N–H).^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 13.9 (CH_3 at piperidin ring), 14.9 (CH_3 at thiadiazole ring), 37.0 (C-5), 46.3 (C-3), 61.2 (C-6), 69.3 (C-2), 126.7–128.8 (Ar–C), 135.0 (C-5 at thiadiazole ring), 142.6 and 142.0 (*ipso* carbons), 159.5 (C-4), 161.9 (NHCO) and 164.6 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-ethyl-2r,6c-diarylpiperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5g). White solid, yield: 70%, mp: 189 °C. IR (KBr, ν_{max} cm^{-1}): 1651 (C═N). ^{1}H-NMR (δ, 400 MHz-$CDCl_3$, ppm): 0.92 (t, 3H, CH_3 at piperidin ring), 1.28 (m, 2H, CH_2 at piperidin ring), 1.90 (s, 1H, NH at piperidin ring), 2.43 (d, 1H, C5-1Ha), 2.68 (s, 3H, methyl at thiadiazole ring), 2.68 (t, 1H, C3-1H), 3.28 (dd, 1H, C5-1He), 3.75 (d, 1H, C2-1H), 3.94 (d, 1H, C6-1H), 7.32–7.48 (m, 10H, Ar–H), 10.14 (s, 1H, N–H, amide NH). ^{13}C (δ, 400 MHz-$CDCl_3$, ppm): 12.41 (CH_3 at piperidin ring), 15.0 (CH_3 at thiadiazole), 19.4 (CH_2 at piperidin ring), 37.3 (C-5), 52.7 (C-3), 61.1 (C-6), 67.8 (C-2), 126.6–128.8 (Ar–C), 135.1 (C-5 at thiadiazole), 142.0 and 142.6 (*ipso* carbons) and 158.1 (C-4), 161.6 (NHCO) and 164.6 (C-4 at thiadiazole).

4-Methyl-*N*′-(3-ethyl-2r,6c-bis(*p*-methylphenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5h). White solid, yield: 65%, m.p: 150 °C. IR (KBr, ν_{max} cm^{-1}): 1651 (C═N). ^{1}H NMR (δ, 400 MHz-$CDCl_3$, ppm): 0.91 (t, 3H, CH_3 at piperidin ring), 1.46 (m, 2H, CH_2 at piperidin ring), 2.01 (s, 1H, NH at piperidin ring), 2.34 (s, 6H, CH_3 at phenyl ring), 2.39 (dd, 1H, C5-1Ha), 2.60 (m, 1H, C3-1H), 2.68 (s, 3H, CH_3 at thiadiazole ring), 3.25 (d, 1H, C5-1He), 3.70 (d, 1H, C2-1H), 3.89 (d, 1H, C6-1H), 7.00–7.36 (m, 8H, Ar–H), 10.16 (s, 1H, N–H amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 12.4 (CH_3 at piperidin ring), 15.0 (CH_3 at thiadiazole ring), 19.4 (CH_2 at piperidin ring), 21.2 (CH_3 at phenyl group), 37.4 (C-5), 52.7 (C-3), 60.9 (C-6), 67.5 (C-2), 126.5–129.3 (Ar–C), 135.1 (C-5 at thiadiazole

ring), 139.1–139.7 (*ipso* carbons), 158.4 (C-4), 161.6 (NHCO) and 164.6 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-ethyl-2r,6c-bis(*p*-methoxyphenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5i). White solid, yield: 73%, m.p: 151 °C. IR (KBr, ν_{max} cm^{-1}): 1649 (C═N). ^{1}H-NMR (δ, 400 MHz-$CDCl_3$, ppm): 0.91 (t, 3H, CH_3 at piperidine ring), 1.46 (m, 2H, CH_2 at piperidin ring), 2.17 (s, 1H, NH at piperidin ring), 2.39 (t, 1H, C5-1Ha), 2.60 (t, 1H, C3-1H), 2.60 (s, 1H, CH_3 at thiadiazole ring), 3.31 (dd 1H, C5-1He), 3.67 (d, 1H, C2-1H), 3.82 (dd, 1H, C6-IH), 3.82 (s, 6H, OCH_3), 7.26–7.39 (m, 8H, Ar–H), 10.60 (s, 1H, N–H amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 12.5 (CH_3 at piperidin ring), 15.0 (CH_3 at thiadiazole ring), 19.4 (CH_2 at piperidin ring), 37.7 (C-5), 53.0 (C-3), 55.3 (OCH_3), 60.6 (C-6), 67.2 (C-2), 113.9–128.9 (Ar–C), 134.3 (C-5 at thiadiazole ring), 134.9 and 135.1 (*ipso* carbons) and 158.9 (C-4), 162.0 (NHCO), and 164.5 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-ethyl-2r,6c-bis(*p*-chlorophenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5j). White solid, yield: 70%, m.p: 169 °C. IR (KBr, ν_{max} cm^{-1}): 1645 (C═N). ^{1}H NMR (δ, 400 MHz-$CDCl_3$, ppm): 0.91 (t, 3H, CH_3 at piperidin ring), 1.45 (m, 2H, CH_2 at piperidin ring), 2.00 (s, 1H, NH at piperidin ring), 2.37 (t, 1H, C5-1Ha), 2.56 (t, 1H, C3-1H), 2.69 (s, 3H, CH_3 at thiadiazole ring), 3.28 (dd, 1H, C5-1He), 3.72 (d, 1H, C2,-1H), 3.91 (dd, 1H, C6-1H), 7.26–7.43 (m, 8H, Ar–H), 10.23 (s, 1H, N–H, amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 12.4 (CH_3 at piperidin ring), 15.0 (CH_3 at thiadiazole ring), 19.4 (CH_2 at piperidin ring), 37.3 (C-5), 52.7 (C-3), 60.4 (C-6), 66.9 (C-2), 128.0–129.2 (arc), 133.9 (C-5 at thiadiazole ring), 141.0 and 140.4 (*ipso* carbons), 157.4 (C-4), 161.9 (NHCO) and 164.5 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-ethyl-2r,6c-bis(*p*-fluorophenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5k). White solid, yield: 78%, m.p: 168 °C. IR (KBr, ν_{max} cm^{-1}): 1641 (C═N). ^{1}H NMR (δ, 400 MHz-$CDCl_3$, ppm): 0.91 (t, 3H, CH_3 at piperidin ring), 1.42 (m, 2H, CH_2 at piperidin ring), 1.99 (s, 1H, NH at piperidin ring), 2.54 (t, 1H, C5-1Ha), 2.57 (t, 1H, C3-1H), 2.65 (s, 3H, CH_3 at thiadiazole ring), 3.29 (dd, 1H, C5-1He), 3.73 (d, 1H, C2-1H), 3.92 (dd, 1H, C6-1H), 7.00–7.46 (m, 8H, Ar–H), 10.38 (s, 1H, N–H, amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 12.4 (CH_3 at piperidin ring), 15.0 (CH_3 at thiadiazole ring), 19.4 (CH_2 at piperidin ring), 37.5 (C-5), 52.9 (C-3), 60.4 (C-6), 66.9 (C-2), 128.3–137.7 (arc), 135.0 (C-5 at thiadiazole ring), 138.4 and 138.4 (*ipso* carbons), 157.8 (C-4), 161.9 (NHCO) and 164.5 (C-4 at thiadiazole ring).

4-Methyl-*N*′-(3-ethyl-2r,6c-bis(*p*-bromophenyl)piperidin-4-ylidene)-1,2,3-thiadiazole-5-carbohydrazide (5l). White solid, yield: 65%, m.p: 177 °C. IR (KBr, ν_{max} cm^{-1}): 1647 (C═N). ^{1}H NMR (δ, 400 MHz-$CDCl_3$, ppm): 0.91 (t, 3H, CH_3 at piperidin ring), 1.27 (m, 2H, CH_2 at piperidin ring), 2.01 (s, 1H, NH at piperidin ring), 2.35 (t, 1H, C5-1Ha), 2.56 (t, 1H, C3-1H), 2.72 (s, 3H, CH_3 at thiadiazole ring), 3.24 (dd, 1H, C5-1He), 3.71 (d, 1H, C2-1H), 3.90 (d, 1H, C6-1H), 7.26–7.52 (m, 8H, Ar–H), 10.07 (s, 1H, N–H amide N–H). ^{13}C NMR (δ, 400 MHz-$CDCl_3$, ppm): 12.4 (CH_3 at piperidin ring), 15.1 (CH_3 at thiadiazole ring), 19.5 (CH_2 at piperidin ring), 37.1 (C-5), 52.6 (C-3), 60.4 (C-6), 69.9 (C-2), 128.3–132.0 (arc), 135.0 (C-5 at thiadiazole ring), 140.8 and 141.4 (*ipso* carbons), 157.0 (C-4), 161.6 (NHCO) and 164.6 (C-4 at thiadiazole ring).

In vitro free radical scavenging assays

The free radical scavenging capacity was evaluated by the DPPH assay described by Blois.[25] The total antioxidant potential was measured by the ABTS assay that measures the relative ability of antioxidants to scavenge the $ABTS^{\bullet+}$ cation radical generated in the aqueous phase.[26] Hydroxyl radical scavenging activity was determined by the method of Halliwell *et al.*[27] on the basis of the ability to compete with deoxyribose for hydroxyl radicals. The nitric oxide radical inhibition activity was evaluated according to the method of Nishimiki *et al.*[28] Superoxide anions derived from dissolved oxygen by a PMS/NADH coupling reaction reduced nitro blue tetrazolium (NBT), which was measured by the method of Garrat using Griess reagent.[29]

Cell culture and maintenance

HeLa cells derived from cervical cancer cells, adenocarcinoma human alveolar basal epithelial cells (A549) and normal lung epithelial cells (NL20) were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in minimum essential medium, Dulbecco's modified Eagle's medium (DMEM), and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Co., St Louis, USA), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) as antibiotics (Himedia, Mumbai, India) under a humidified atmosphere of 5% CO_2 and 95% air in a CO_2 incubator.

Cell viability assay by MTT

Cell survival was assessed by the MTT assay. HeLa, A549 and NL20 cells, grown to approximately 80% confluence, were trypsinized, counted, seeded in 96-well plates with an average population of 1000 cells per well, incubated overnight, and then treated for 24 h with compounds **5a–5l** (0, 3, 6, 9, 12, 15, and 18 μM). All experiments were done using three replicates. Untreated cells were used as controls.

Cell proliferation assay by the crystal blue staining method

We have treated the A549 cells with synthetic compounds **5e** and **5k** at 2.5, 5, and 10 μM. Compounds **5e** and **5k** at 5 and 10 μM showed a greater inhibitory effect on A549 cells and the medium dose of 5 μM was more effective when compared to other doses and MMC, the standard anticancer agent. Therefore, we have used 5 and 10 μM doses of the synthetic compounds **5e** and **5k** for crystal violet blue staining and cell migration assay. A549 and NL20 cells, grown to approximately 80% confluence, were trypsinized, counted, and seeded in 12-well plates with an average population of 4000 cells per well, incubated overnight, and then treated for 24 h with compounds **5e** and **5k** (5 and 10 μM). All experiments were done using three replicates. Untreated cells were used as controls. MMC (50 ng ml^{-1}) was used as a positive control.

IC_{50} values for the synthetic compounds **5e** and **5k** in A549 and NL20 cells was also confirmed using the crystal blue staining assay by the method of Itagaki *et al*.[30] A549/NL20 cells were seeded in 96-well microplates which contained serially diluted synthetic compounds **5e** and **5k** (0, 1.5/3, 3/6, 6/9, 9/12, 12/15, 15/18, and 18.21 μM). The cells were incubated for 24 h, and then fixed, stained with crystal violet. The cells were washed with PBS two times and a third to half the total well volume of straight methanol was added to solubilize the dye. The intensity of the dye colours was read at 540 nm. After spectrophotometric measurement, the concentrations of test materials that inhibited the absorbance to 50% of the control level (IC_{50}) were determined. All experiments were done using three replicates. Untreated cells were used as controls.

Cell migration assay

HeLa cells were grown in 5 μM **5e** and **5k** for 24 h and plated into a chamber with transwells the following day in a medium containing 5 μM **5e** and **5k** in triplicate as described in the procedure provided by the manufacturer. The chamber transwell was taken out of the chamber 2 h after seeding, and was fixed and stained with DAPI. The number of cells per microscope field was calculated by averaging 10 fields randomly selected.

In vitro antibacterial and antifungal activity by the twofold serial dilution method

Bacterial strains such as *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, and fungal strains such as *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans* and *Rhizopus* obtained from the Faculty of Medicine, Annamalai University, Annamalai nagar 608 002, Tamil Nadu, India, were used to screen the antimicrobial activity of the newly synthesized compounds **5(a–l)**. The bacterial and fungal strains were cultured in Sabouraud dextrose broth (SDB) at pH 7.4 ± 0.2 (Hi-media, Mumbai) and nutrient broth (NB) (Hi-media, Mumbai) at pH 5.6 respectively.

The *in vitro* potency of compounds **5(a–l)** was examined by the twofold serial dilution method.[31] Stock solutions of **5(a–l)** were made in DMSO (1 mg ml^{-1}). Compounds were tested in the concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.12 μg ml^{-1} (twofold serial dilution) with SDB and NB. Then SDB and NB were suspended with 100 μL of bacterial spores from 24 h old bacterial cultures on NB at 37 ± 1 °C and 100 μL fungal spores from 1 to 7 day old SDB slant cultures at 28 ± 1 °C. Plating techniques were used to determine the colony forming units (cfu) of the seeded broth in the adjusted range of 104–10^5 cfu ml^{-1}. 10^5 cfu ml^{-1} and 1.1–1.5 × 10^2 cfu ml^{-1} were the final inoculum sizes for antibacterial and antifungal assays, respectively. Microbial spore supplemented broth with DMSO at the highest concentrations used in our experiments was used as the negative control. The growth of the microbes in the test medium was measured based on the turbidity of the culture after 24 h of bacterial incubation and 72–96 h of fungal incubation. The lowest concentration of the test compounds with the clear solutions of test medium was considered to be the minimum inhibitory concentration (MIC). Drug standards are streptomycin for anti-bacterial activity and fluconazole for fungal studies.

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