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Synthesis and anticancer activities of novel 3,5-disubstituted-1,2,4-oxadiazoles

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ABSTRACT

A series of 3,5-disubstituted-1,2,4-oxadiazoles were synthesized and evaluated for their in vitro anti-proliferative activities against various cancer cell lines. Formation of 1,2,4-oxadiazole ring was accomplished by the reaction of amidoxime with carboxylic acids. The in vitro cytotoxic effects of 3,5-disubstituted-1,2,4-oxadiazoles have been demonstrated across a wide array of tumor cell types and a few compounds exhibited specificity towards pancreatic (**3f**, **3h**, **3j**, and **3k**) and prostate (**3n**) cancer cells. Among the prepared 3,5-disubstituted-1,2,4-oxadiazoles, compound **3n** is the most selective (>450-fold) and compound **3p** is the most cytotoxic (10 nM) against prostate cancer cell lines.

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Recently, there has been wide interest in compounds containing the 1,2,4-oxadiazole scaffold because of their unique chemical structure and broad spectrum of biological properties including tyrosine kinase inhibition,[1] muscarinic agonism,[2] histamine H3 antagonism,[3] anti-inflammation,[4] antitumor,[5] and monoamine oxidase inhibition.[6] The 1,2,4-oxadiazoles are also widely used as heterocyclic amide or ester bioisosters[7] and in the design of dipeptidomimetics as peptide building blocks.[8,9]

Cancer is a fatal disease that has posed serious threat to human health. Many classes of anticancer drugs have been developed. However, most drugs cause undesirable side effects due to lack of tumor specificity and multidrug resistance. Several factors aid in cancer cell survival. These include variation in metabolism, absorption and delivery of drug to target tissues, and tumor location. For example, chemotherapy agents such as paclitaxel, vincristine, and doxorubicin have lost their efficacy due to the development of drug resistance from prolonged exposure. Compounds such as epothilone and discodermolide exhibit activities against multi-drug resistant cancer cells and have been evaluated in clinical trials. Recently, ixabepilone, an epothilone derivative was approved for breast cancer in the US. Several other epothilone derivatives are in advanced clinical trials. Discodermolide, however, proved toxic and has been dropped.[10] Therefore, the development of novel and effective anticancer agents remains a major challenge. In this letter, we report the synthesis of novel 3,5-disubstituted-1,2,4-oxadiazoles and measure their anticancer activities in various cancer cell lines.

The 3,5-disubstituted-1,2,4-oxadiazoles **3** were synthesized according to Scheme 1. The reaction of appropriate benzonitrile **1** with hydroxylamine hydrochloride under refluxing condition afforded amidoxime **2**[11a] which was coupled with the corresponding carboxylic acid using DCC in DMF to give 3,5-disubstituted-1,2,4-oxadiazoles **3** in moderate yield.[11b] All synthesized compounds were characterized by NMR and mass spectroscopy.[12]

A library of 14 compounds was tested in vitro for their anticancer potential in various human cancer cell lines: prostate (PC3, DU145 and LnCaP), breast (MCF7 and MDA231), colon (HCT116), and pancreas (PaCa2).[13]

All compounds decreased cell viability as determined by colorimetric MTT assay, with IC_{50} values ranging from nanomolar to greater than 1 mM (Table 1). More importantly, a few compounds were highly potent and exhibited specificity towards one cell type relative to others.

The activities of various 3,5-disubstituted-1,2,4-oxadiazoles revealed **3f**, **3g**, **3n**, and **3p** as potent cytotoxic agents against various cancer cell lines (IC_{50} ranging from 10 nM to 860 nM). All of these compounds possess common 1,2,4-oxadiazole nucleus in which substitution at C-3 and C-5 positions play important roles in potency and selectivity of 3,5-disubstituted-1,2,4-oxadiazoles **3**. Compounds **3a** and **3b**, with C-3 phenyl and C-5 4-fluorophenyl/pyridin-4-yl groups, displayed weak inhibitory activity against tested cancer cell lines.

Introduction of 3′-cyclopentyloxy and 4′-methoxy moieties at C-3 aryl ring enhances the activity (**3e**, **3h** in comparison to **3a**, **3b**, respectively). However, a larger group like benzyloxy at para position of C-3 aryl ring (**3c**) is detrimental to the activity. Replace-

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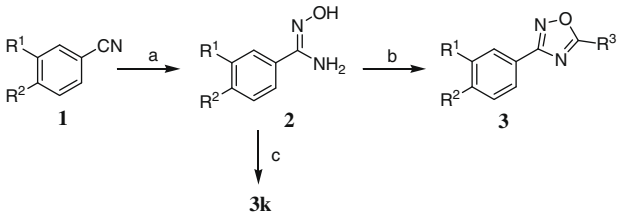


3a: $R^1 = R^2 = H$, $R^3 = 4\text{-}FC_6H_4$
3b: $R^1 = R^2 = H$, $R^3 =$ pyridin-4-yl
3c: $R^1 = OCH_3$, $R^2 = OBn$, $R^3 =$ pyridin-4-yl
3d: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 = C_6H_5$
3e: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 = 4\text{-}FC_6H_4$
3f: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 = 4\text{-}ClC_6H_4$
3g: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 = 4\text{-}HOC_6H_4$
3h: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 =$ pyridin-4-yl
3i: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 =$ pyridin-3-yl
3j: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 =$ (indol-3-yl)methyl
3k: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 = CH_2COCH_3$
3l: $R^1 = OH$, $R^2 = OCH_3$, $R^3 = 4\text{-}FC_6H_4$
d **3m**: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 =$ N-Boc-pyrrolidin-2-yl
3n: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 =$ pyrrolidin-2-yl
d **3o**: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 =$ N-Boc-piperidin-4-yl
3p: $R^1 =$ cyclopentyloxy, $R^2 = OCH_3$, $R^3 =$ piperidin-4-yl

Scheme 1. Synthesis of 1,2,4-oxadiazoles. Reagents and conditions: (a) EtOH, H_2O, $NH_2OH.HCl$, NaOH, reflux, 18 h; (b) DMF, DCC, aromatic/heteroaromatic/aliphatic carboxylic acid, 0 °C to 30 °C, 3 h then 110 °C 12 h; (c) ethylacetoacetate, reflux; (d) trifluoroacetic acid, DCM.

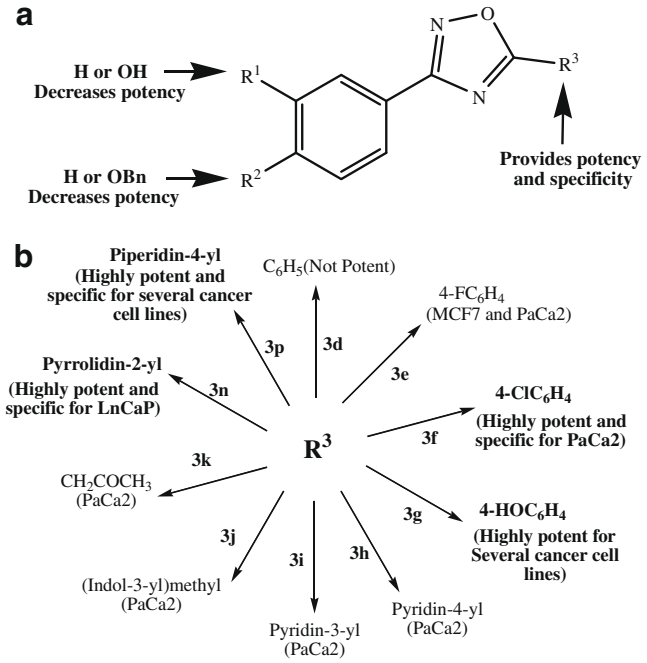
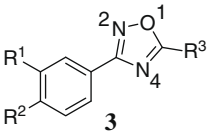


Figure 1. SAR of 1,2,4-oxadiazoles (a) Substitution at R^3 provides both specificity and potency. (b) In this panel, $R^1 = OC_5H_9$ and $R^2 = OCH_3$. Phenyl or Pyrindin-3-yl substitution at R^3 position decreases potency. CH_2COCH_3, pyrindin-4-yl or (Indol-3-yl)methyl at R^3 position confer specificity for PaCa2 cells (IC_{50} 3.0–9.56 μM). Substituents at R^3 shown in bold confer high potency. While compounds containing $4\text{-}HOC_6H_5$ and piperidin-4-yl substituents at R^3 position are highly cytotoxic in multiple cancer cell lines, both $4\text{-}ClC_6H_5$ and pyrrolidin-2-yl are highly potent and specific for PaCa2 and LnCaP cells, respectively.

ment of 4-fluorophenyl in **3a** with 4-chlorophenyl or 4-hydroxyphenyl group yielded compounds **3f** and **3g**, respectively, with significant improved activities against different cancer cell lines. Compounds **3f** with 4-chlorophenyl, and **3h** with pyridin-4-yl groups show selectivity against pancreatic cancer cell line with IC_{50} values of 0.74 μM and 9.33 μM, respectively. Significant loss in activity was observed upon changing the position of nitrogen atom in the C-5 pyridyl ring of compound **3h** to obtain the isomer **3i**. When C-5 aryl ring is replaced with an aliphatic ketone moiety as represented by **3k**, improved cytotoxicity was observed. Interestingly, compound **3k** also exhibited selective cytotoxicity against pancreatic cancer cell line (3.01 μM). Replacement of C-5 phenyl group of **3d** with pyrrolidin-2-yl yielded compound **3n**, which exhibited high potency as well as selectivity against prostate cancer cell line (LnCaP, 43 nM). Substitution with piperidin-4-yl group in **3d** at C-5 position led to the compound **3p** with significant improved activity against most cancer cell lines and 6-fold selectivity against LnCaP (10 nM), a prostate cancer cell line.

The compound **3j** with an (indol-3-yl)methyl group at the C-5 position was also selectively cytotoxic towards pancreatic

Table 1
Cytotoxicity profile of 3,5-disubstituted-1,2,4-oxadiazoles against selected human cancer cell lines, IC_{50} (μM)



Compd	R^1	R^2	R^3	PC3	DU145	LnCaP	MCF7	MDA231	HCT 116	PaCa2
3a	H	H	$4\text{-}FC_6H_4$	$>10^3$	$>10^3$	23.7 ± 0.5	$>10^3$	$>10^3$	$>10^3$	$>10^3$
3b	H	H	Pyridin-4-yl	$>10^3$	656.6 ± 0.1	710 ± 0.5	$>10^3$	$>10^3$	$> 10^3$	497 ± 0.09
3c	OCH_3	OBn	Pyridin-4-yl	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$
3d	OC_5H_9	OCH_3	C_6H_5	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$
3e	OC_5H_9	OCH_3	$4\text{-}FC_6H_4$	>103	20.65 ± 0.1	87.93 ± 0.5	3.88 ± 0.3	55.47 ± 0.1	209.7 ± 0.6	6.09 ± 0.08
3f	OC_5H_9	OCH_3	$4\text{-}ClC_6H_4$	$>10^3$	122.5 ± 0.1	20.8 ± 0.4	174.2 ± 0.1	395.5 ± 0.1	$>10^3$	**0.74** ± 0.02
3g	OC_5H_9	OCH_3	$4\text{-}HOC_6H_4$	162.3 ± 0.08	**0.78** ± 0.08	**0.86** ± 0.05	**0.39** ± 0.3	**0.31** ± 0.05	**0.27** ± 0.05	**0.48** ± 0.08
3h	OC_5H_9	OCH_3	Pyridin-4-yl	$>10^3$	$>10^3$	$>10^3$	$>10^3$	240.1 ± 0.1	$>10^3$	9.33 ± 0.06
3i	OC_5H_9	OCH_3	Pyridin-3-yl	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$
3j	OC_5H_9	OCH_3	(Indol-3-yl)methyl	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	9.56 ± 0.08
3k	OC_5H_9	OCH_3	CH_2COCH_3	$>10^3$	302.1 ± 0.1	28.34 ± 0.4	174.2 ± 0.1	53.3 ± 0.1	184.8 ± 0.3	3.01 ± 0.03
3l	OH	OCH_3	$4\text{-}FC_6H_4$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	350.7 ± 0.09
3n	OC_5H_9	OCH_3	Pyrrolidin-2-yl	$>10^3$	242 ± 0.1	**0.043** ± 0.04	$>10^3$	212.2 ± 0.3	184.8 ± 0.1	19.49 ± 0.08
3p	OC_5H_9	OCH_3	Piperidin-4-yl	$>10^3$	**0.057** ± 0.07	**0.010** ± 0.02	3.88 ± 0.3	**0.37** ± 0.08	1.54 ± 0.03	**0.54** ± 0.03

These experiments were conducted in triplicates at three independent times. IC50 values were obtained using a dose response curve by nonlinear regression using a curve fitting program, GraphPad Prism 5.0. Bold values show IC_{50} of less than 1 μM. OC_5H_9 = Cyclopentyloxy.

(9.56 μM) cancer cell line. Removal of 3′-cyclopentyl group in compound **3e** led to the compound 3l having 3′-hydroxy-4′-methoxyphenyl group at C-3 position, which results in complete loss of activity. Aryl/Heteroaryl substituents with a polar functionality at C-5 and 3′-cyclopentyloxy-4′-methoxyphenyl moiety at C-3 position of 1,2,4-oxadiazole core is very important for the activity and selectivity. The observed structure–activity relationship is illustrated in Figure 1. So far, molecular targets responsible for the observed cytotoxicity of this new series of 3,5-disubstituted-1,2,4-oxadiazoles **3** have not been identified, and a reasonable explanation of the substitutions described above is not yet possible. Among synthesized 3,5-disubstituted-1,2,4-oxadiazoles, compounds **3f**, **3g**, **3n**, and **3p** were most potent and selective in the in vitro assay.

In summary, our SAR study shows that the 3,5-disubstituted-1,2,4-oxadiazoles decrease cell viability in various cancer cell lines with IC_{50} values ranging from 10 nM to >1 mM. While **3n** was highly specific and potent for LnCaP cells, a few others (**3f**, **3h**, **3j**, and **3k**) exhibited specificity towards pancreatic cell line. The substituents at C-3 and C-5 positions of 1,2,4-oxadiazole ring were shown to be vital for potency, suggesting specific interactions of these groups with biological targets. Extensive exploration of structure–activity relationship of this novel 1,2,4-oxadiazole scaffold and its biological target studies are underway.

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11. (a) *Synthesis of amidoxime* **2**: To a mixture of appropriate benzonitrile (10 mmol) and hydroxyl-amine hydrochloride (20 mmol) in 50 mL of ethanol was added dropwise aqueous solution of sodium hydroxide (20 mmol, 10 mL) while maintaining temperature at 0 °C. The resulting mixture was allowed to reflux with stiring for 18 h. Ethanol was distilled off under reduced pressure and the crude product was taken into 50 mL of water. The pH (~2) of the solution was adjusted with 1N HCl and the aqueous phase was washed with ethylacetate (2 × 25 mL). Upon cooling (0 °C) and neutralization with sodium carbonate gave off white precipitate which was filtered, washed and air dried at 60 °C to afford pure amidoxime **2**.; b *Synthesis of 3,5-disubstituted-1,2,4-oxadiazole* **3**: A solution of appropriate carboxylic acid (0.8 mmol) in dry DMF (1 mL) was cooled to 0 °C and added dicyclohexylcarbodiimide (1.2 mmol) under nitrogen atmosphere and stirred the reaction mixture at the same temperature for 1 h. To this reaction mixture appropriate benzamidoxime **2** (0.8 mmol) was added and stirred at 0 °C for 0.5 h. The reaction mixture was slowly brought to 30 °C, and stirring continued for another 3 h. Gradually temperature was raised to 110 °C, and further stirred for 10 h. The reaction mixture was cooled to 25 °C and poured into ice-cold water (25 mL). Upon addition of ethylacetate (25 mL) and stirring for 10 min, crystals of dicyclohexylurea separated out and removed by filtration. Separated aqueous phase was extracted with ethylacetate (2 × 20 mL) and the combined organic phase was washed with brine, dried over anhydrous sodium sulfate. Ethylacetate was distilled off and the residue thus obtained was purified by flash column chromatography using ethylacetate–hexane (0–25%) as eluent to afford pure oxadiazole **3**.
12. *Data for selected compounds* **3a**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.26–8.23 (m, 2H), 8.18–8.16 (m, 2H), 7.54–7.50 (m, 3H), 7.27–7.23 (m, 2H). Compound **3b**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.89 (dd, 2H, *J* = 4.48, 1.64 Hz), 8.19–8.17 (m, 2H), 8.07 (dd, 2H, *J* = 4.44, 1.68 Hz), 7.56–7.52 (m, 3H). Compound **3c**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.88 (d, 2H, *J* = 5.0 Hz), 8.06 (dd, 2H, *J* = 4.56, 1.56 Hz), 7.72 (dd, 1H, *J* = 8.36, 2.0 Hz), 7.67 (d, 1H, *J* = 1.96 Hz), 7.46 (d, 2H, *J* = 8.76 Hz), 7.41–7.32 (m, 3H), 7.00 (d, 1H, *J* = 8.4 Hz), 5.25 (s, 2H), 4.00 (s, 3H). Compound **3d**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.22 (dd, 2H, *J* = 5.20, 0.9 Hz), 7.76 (dd, 1H, *J* = 6.24, 1.44 Hz), 7.67 (d, 1H, *J* = 1.44 Hz), 7.61–7.54 (m, 3H), 6.97 (d, 1H, *J* = 6.3 Hz), 4.94–4.91 (m, 1H), 3.92 (s, 3H), 2.17–1.84 (m, 6H), 1.67–1.62 (m, 2H). Calcd *m/z* for $C_{20}H_{20}N_2O_3$: 336.1, found: 337.2 $(M+H)^+$, 359.3 (M+Na). Compound **3e**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.25–8.21 (m, 2H), 7.74 (dd, 1H, *J* = 8.36, 2.04 Hz), 7.66 (d, 1H, *J* = 1.92 Hz), 7.27–7.22 (m, 2H), 6.97 (d, 1H, *J* = 8.4 Hz), 4.93–4.90 (m, 1H), 3.92 (s, 3H), 2.04–1.84 (m, 6H), 1.67–1.62 (m, 2H). Calcd *m/z* for $C_{20}H_{19}FN_2O_3$: 354.1, found: 355.2 $(M+H)^+$, 377.2 (M+Na). Compound **3f**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.16 (d, 2H, *J* = 8.64 Hz), 7.74 (dd, 1H, *J* = 8.40, 1.92 Hz), 7.56 (d, 1H, *J* = 1.92 Hz), 7.53 (d, 2H, *J* = 8.64 Hz), 6.97 (d, 1H, *J* = 8.40 Hz), 4.93–4.90 (m, 1H), 3.92 (s, 3H), 2.06–1.84 (m, 6H), 1.67–1.60 (m, 2H). Compound **3g**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.11 (d, 2H, *J* = 8.80 Hz), 7.74 (dd, 1H, *J* = 8.28, 1.92 Hz), 7.66 (d, 1H, *J* = 1.92 Hz), 7.01–6.95 (m, 3H), 6.50 (s, 1H (OH)), 4.93–4.88 (m, 1H), 3.91 (s, 3H), 2.04–1.86 (m, 6H), 1.65–1.56 (m, 2H). Compound **3h**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.88 (dd, 2H, *J* = 4.48, 1.68 Hz), 8.06 (dd, 2H, *J* = 4.08, 1.68 Hz), 7.76 (dd, 1H, *J* = 8.40, 2.04 Hz), 7.66 (d, 1H, *J* = 1.96 Hz), 6.98 (d, 1H, *J* = 8.40 Hz), 4.94–4.90 (m, 1H), 3.93 (s, 3H), 2.06–1.85 (m, 6H), 1.68–1.61 (m, 2H). Calculate *m/z* for $C_{19}H_{19}N_3O_3$: 337.1, found: 338.2 $(M + H)^+$, 360.2 (M+Na). Compound **3i**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 9.45 (dd, 1H, *J* = 2.08, 0.56 Hz), 8.84 (dd, 1H, *J* = 4.88, 1.68 Hz), 8.48 (dt, 1H, *J* = 8.04, 2.0 Hz), 7.76 (dd, 1H, *J* = 8.4, 2.0 Hz), 7.67 (d, 1H, *J* = 1.92 Hz), 7.52 (ddd, 1H, *J* = 8, 4.8, 0.6 Hz), 6.98 (d, 1H, *J* = 8.44 Hz), 4.93–4.92 (m, 1H), 3.93 (s, 3H), 2.04–1.85 (m, 6H), 1.71–1.62 (m, 2H), Calcd *m/z* for $C_{19}H_{19}N_3O_3$: 337.1, found: 338.2 $(M+H)^+$, 360.2 (M+Na). Compound **3j**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.22 (s, 1H), 7.70 (d, 1H, *J* = 8.84 Hz), 7.64 (dd, 1H, *J* = 8.32, 1.92 Hz), 7.57 (d, 1H, *J* = 1.96 Hz), 7.37 (d, 1H, *J* = 8.08 Hz), 7.24–7.13 (m, 3H), 6.91 (d, 1H, *J* = 8.44 Hz), 4.87–4.84 (m, 1H), 4.43 (s, 2H), 3.88 (s, 3H), 2.04–1.80 (m, 6H), 1.67–1.59 (m, 2H). Calcd *m/z* for $C_{23}H_{23}N_3O_3$: 389.2, found: 390.3 $(M+H)^+$, 412.3 (M+Na). Compound **3k**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 7.65 (dd, 1H, *J* = 8.40, 2.04 Hz), 7.57 (d, 1H, *J* = 1.96 Hz), 6.94 (d, 1H, *J* = 8.44 Hz), 4.89–4.86 (m, 1H), 4.1 (s, 2H), 3.91 (s, 3H), 2.35 (s, 3H), 2.17–1.80 (m, 6H), 1.64–1.58(m, 2H). Calcd *m/z* for $C_{17}H_{20}N_2O_4$: 316.1, found: 317.3 $(M+H)^+$, 339.3 (M+Na). Compound **3l**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 8.24–8.20 (m, 2H), 7.73–7.69 (m, 2H), 7.23 (d, 2H, *J* = 4.6 Hz), 6.96 (d, 1H, *J* = 8.20 Hz), 5.72 (s, 1H (OH)), 3.97 (s, 3H). Compound **3n**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 7.66 (dd, 1H, *J* = 8.36, 2.0 Hz), 7.57 (d, 1H, *J* = 1.96 Hz), 6.93 (d, 1H, *J* = 8.44 Hz), 4.89–4.87 (m, 1H), 4.56–4.52 (m, 1H), 3.90 (s, 3H), 3.24–3.20 (m, 1H), 3.12–3.08 (m, 1H), 2.33–2.29 (m, 1H), 2.17–1.80 (m, 8H), 1.64–1.58(m, 2H). Calcd *m/z* for $C_{18}H_{23}N_3O_3$: 329.2, found: 330.3 $(M+H)^+$, 352.3 (M+Na). Compound **3o**: ^{1}H NMR ($CDCl_3$, 200 MHz): δ_H = 7.63 (dd, 1H, *J* = 8.00, 2.00 Hz), 7.55 (d, 1H, *J* = 2.00 Hz), 6.91(d, 1H, *J* = 8.00 Hz), 4.89–4.78 (m, 1H), 4.16–4.09 (m, 2H), 3.89 (s, 3H), 3.19–2.87 (m, 2H), 2.14–1.85 (m, 10H), 1.70–1.55 (m, 3H), 1.46 (s, 9H). Compound **3p**: ^{1}H NMR ($CDCl_3$, 200 MHz): δ_H = 7.63 (dd, 1H, *J* = 8.00, 2.00 Hz), 7.54 (d, 1H, *J* = 2.00 Hz), 6.92 (d, 1H, *J* = 8.00 Hz), 4.86–4.78 (m, 1H), 3.88 (s, 3H), 3.40–2.87 (m, 9H), 2.16–1.61 (m, 8H), Calcd *m/z* for $C_{19}H_{25}N_3O_3$: 343.2, found: 344.2 $(M+H)^+$.
13. Cells were cultured in RPMI-1640 media supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin/streptomycin. For MTT assay, cells were seeded in 96 well plates at a density of 4.0×10^3 cells per well for 12 h. Cells were incubated with various concentrations of the compounds ranging from 10 nM–1 mM. After 24 h, MTT (3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazoliumbromide) was added to the final concentration of 0.5 mg/ml and incubated for 30 min. The cells were washed twice with PBS and lysed in dimethylsulfoxide, and the absorbance was measured at 570 nm.