

Synthesis, *In vitro* anticancer activity and molecular docking studies on some new phenylmorpholine linked aminotetrazoles and aryl tetrazoles

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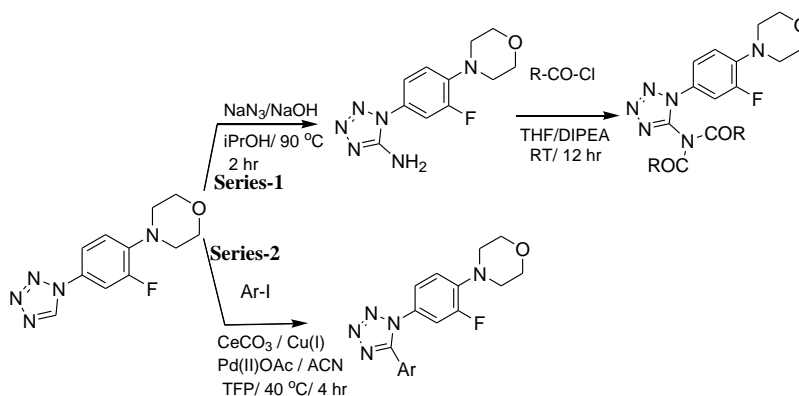
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Abstract

This article presents two new series of tetrazole and morpholine hybrid derivatives that were produced using simple processes and good yields from 2-difluoro-4-nitrobenzene, morpholine, acid chlorides/(or) aryl iodide. By using IR, NMR, and HRMS spectrometry, all compounds were identified. The synthesized compounds were tested for anticancer efficacy against histiocytic lymphoma (U-937) cells. The route of action was investigated using molecular docking against tubulin, and **8h** and **10k** showed the best interactions with tubulin of all the drugs tested.



Keywords: Tetrazole, Morpholine, Hybrid molecules, Synthesis, Analysis, Anticancer efficacy, Molecular docking

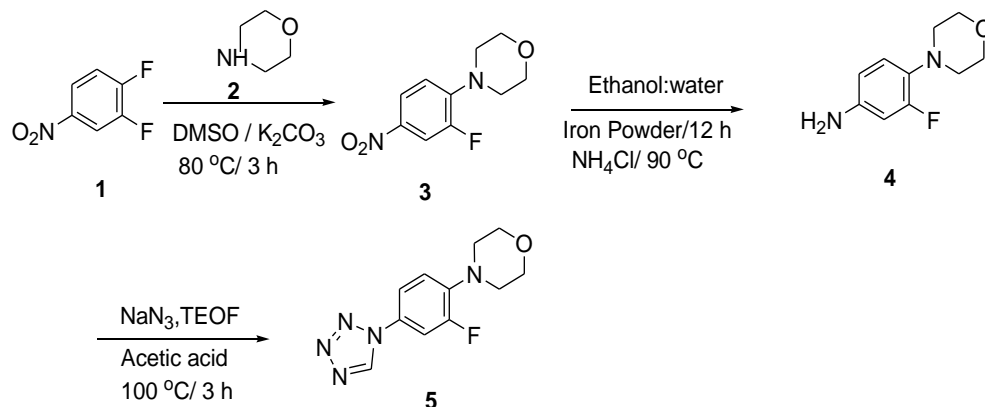
Introduction

A significant family of N-heterocyclic molecules with several applications were tetrazole compounds.¹ Tetrazoles were widely used in many different industries, for example, as stabilizers in photography and photo imaging,² chelating agents in coordination chemistry,³ explosives in rocket propellants,⁴ anti-wear and frictional agents in lubricants,⁵ plant growth regulators, herbicides, and fungicides in agriculture. Tetrazole substances may also be utilized as bio-isosteres for carboxylic acids.⁶ The bioavailability, metabolic stability, and cell permeability of a pharmacological molecule are all significantly increased when a carboxyl group is substituted with a tetrazole moiety. Tetrazole compounds were consequently readily available as a variety of well-known drugs like Candesartan, Zolarsartan,⁸ Losartan,⁹ and Valsartan.¹⁰ In addition, tetrazole compounds showed varied biological activities such as antibacterial,¹¹ antihypertensive,¹² anticonvulsant,¹³ antifungal,¹⁴ anticancer,¹⁵ anti-inflammatory,¹⁶ antitubercular,¹⁷ antiallergic,¹⁸ antineoplastic,¹⁹ and antiviral²⁰ especially anti-HIV²¹ activities.

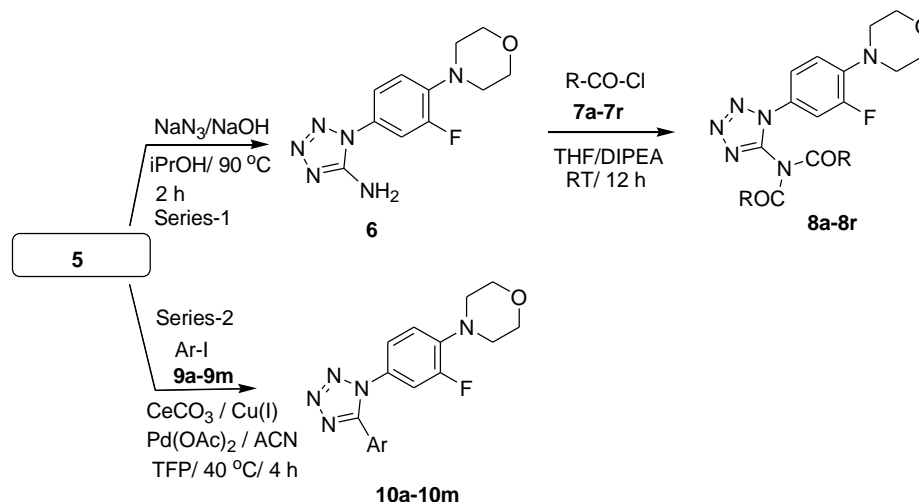
Morpholine key motif is present in many pharmaceutical drugs including Phenadoxone, Timolol, Linezolid²², Reboxetine²³, Moclobemide²⁴, Rivaroxaban²⁵ etc. Morpholine derivatives have a variety of biological activities, including those that are anti-cancer, anti-bacterial, anti-fungal, and antiviral.²⁶ In this article, we present the synthesis of two series of novel tetrazole and morpholine hybrid derivatives as depicted in Schemes 1 and 2.

Results and Discussion

As illustrated in Scheme-1, the key intermediate (**5**) has been synthesized for generating the two series of 4-(2-fluoro-4-(1*H*-tetrazol-1-yl)phenyl) morpholine derivatives. Initially, the commercially easily accessible starting compounds 1,2-difluoro-4-nitrobenzene **1** was condensed with morpholine **2** in the presence of K₂CO₃ as a base in DMSO as solvent at 80 °C for 3 hr to form 4-(2-fluoro-4-nitrophenyl)morpholine **3** with 84% yield. Then, compound **3** underwent reduction with iron powder and ammonium chloride in a mixture of ethanol and water as solvent at 90 °C for 12 h to give 3-fluoro-4-morpholinoaniline **4** with 86% yield. Further, compound **4** was treated with sodium azide in acetic acid in the presence of triethyl orthoformate (TEOF) at 100 °C for 3 h to obtain 4-(2-fluoro-4-(1*H*-tetrazol-1-yl)phenyl)morpholine **5** with 87% yield.



Scheme 1. Synthesis of key intermediate **5**.



Scheme 2. Synthesis of 4-(2-fluoro-4-(1*H*-tetrazol-1-yl)phenyl)morpholine derivatives.

The resulting key intermediate **5** has been used for the synthesis of two series of new tetrazole derivatives. As illustrated in Scheme 2, compound **5** undergoes amination with the help of NaN_3 , NaOH and Et_3N in *i*-PrOH followed by treatment with DMSO in acetic acid at $90\text{ }^\circ\text{C}$ for 2 h to give 1-(3-fluoro-4-morpholinophenyl)-1*H*-tetrazol-5-amine **6** with 83% yield. Then, compound **6** underwent acetylation with different acid chlorides (**7**) in the presence of DIPEA as a base and THF as solvent at RT for 12 h to form **series-1** of tetrazole derivatives [diacylated tetrazoles (using 3 equiv. of acetyl chloride) & mono acetylation tetrazole compounds (using 1.1 equiv. of acetyl chloride)]. On the other hand, compound **5** underwent a coupling reaction with aryl iodide in the presence of cesium carbonate, copper(I) iodide, palladium(II) acetate, and tris(2-furyl)-phosphine in dry acetonitrile at $40\text{ }^\circ\text{C}$ in argon atmosphere for 4 h to form **series-2** of tetrazole derivatives (aryl tetrazole compounds). The structures of the synthesized compounds were ascertained by IR, NMR and mass analysis.

In the scenario, we have synthesized two classes of 1*H*-tetrazol-1-yl)phenyl)morpholine amide derivatives by monoacetylation and di-acetylation. The yields of the compounds were obtained in the range of 64-94%. The representative compound cyclopropanediamide **8d** obtained the highest yield (94%) and the di(trifluoromethyl)benzamide **8h** obtained with lowest yield (64%). All the tetrazolyl phenyl morpholine derivatives (**8a-8r**) were characterized by spectral data. The IR spectral data revealed that compounds (**8a-8j**) showed a strong C=O stretching band at around $1705\text{-}1744\text{ cm}^{-1}$ and $^1\text{H-NMR}$ chemical shift value of **8a** observed in the range of δ 2.30 as singlet for 6 H's revealed that the presence of diacetylated product on amine. Further, the absence of -NH_2 bands in IR spectra (**6**) at $3340, 3154\text{ cm}^{-1}$ also confirmed that smooth di-acetylation underwent on **6** and achieved **8a-8j** in very good yields. Furthermore, the monoacetylated compounds (**8k-8r**) also characterized by spectral data. The IR spectral data revealed that the aforementioned compounds showed a strong C=O stretching band at about $1677\text{-}1728\text{ cm}^{-1}$ and a strong -NH absorption band at $3181\text{-}3405\text{ cm}^{-1}$. ^{13}C NMR and high-resolution mass spectral data (HRMS) also corroborated the structures of the all the morpholine amide derivatives (**8a-8r**).

Additionally, we have synthesized arylated tetrazol-morpholine derivatives (**10a-10m**) by adopting a coupling reaction^{27,28} of compound **5** with various aryl iodides (**9a-9m**) in the presence of palladium(II) acetate and tris(2-furyl)-phosphine with good yields (78-87%). Additional $^1\text{H-NMR}$ chemical shift values at δ at 7.57–7.41 as multiplet for 5 H's of representative compound **10a** and its mass spectra showed m/z at 326 as $[\text{M}+\text{H}]^+$ also revealed that arylation takes place on tetrazole core of compound **5**. ^1H singlet peak at chemical shift

Table 1. Continued

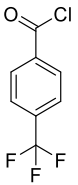
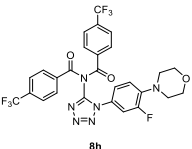
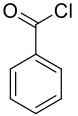
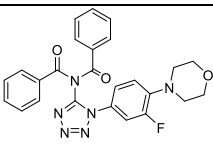
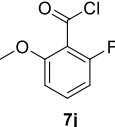
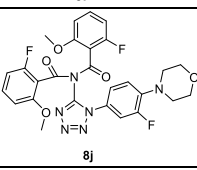
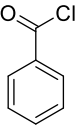
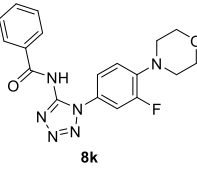
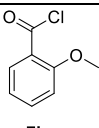
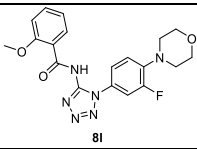
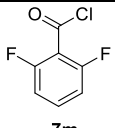
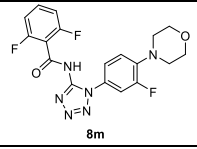
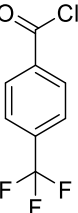
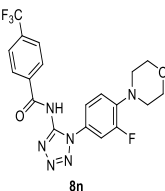
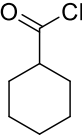
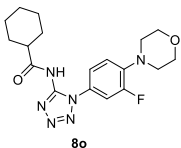
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9	 7i	 8i	101-104	77
10	 7j	 8j	239-241	87
11	 7k	 8k	257-259	85
12	 7l	 8l	196-198	92
13	 7m	 8m	214-216	90
14	 7n	 8n	202-204	87
15	 7o	 8o	201-205	85

Table 1. Continued

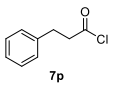
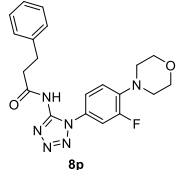
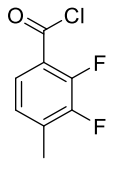
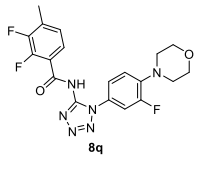
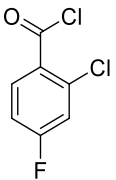
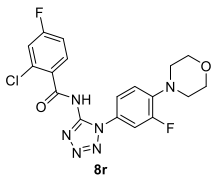
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16	 7p	 8p	180-183	92
17	 7q	 8q	112-115	91
18	 7r	 8r	172-175	88

Table 2. Yield and Melting point details of compounds obtained in 10(a-m)series

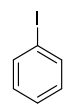
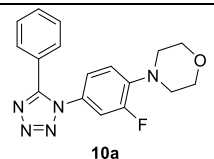
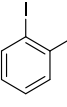
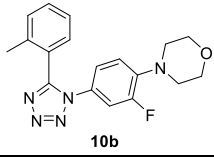
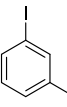
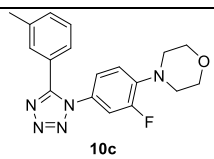
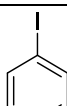
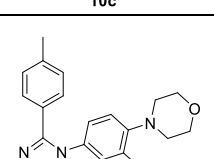
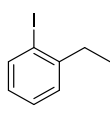
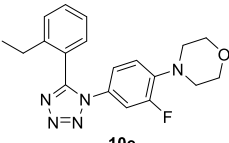
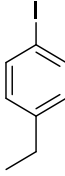
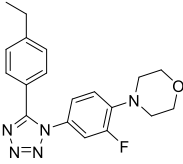
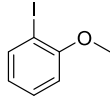
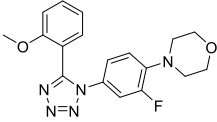
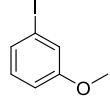
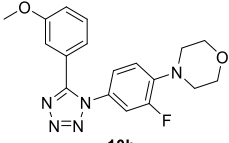
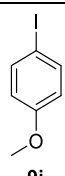
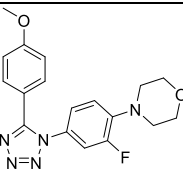
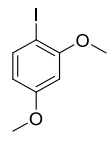
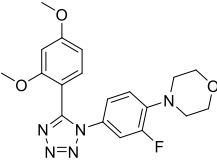
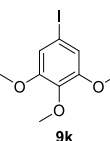
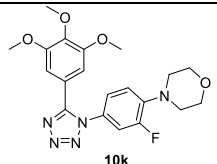
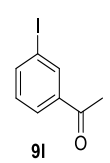
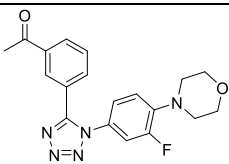
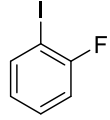
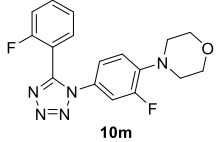
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1	 9a	 10a	148-151	87
2	 9b	 10b	98-101	86
3	 9c	 10c	101-104	83
4	 9d	 10d	149-151	84

Table 2. Continued

Entry	Aryl Iodide	Product	Melting range (°C)	Yield (%)
5	 9e	 10e	--	81
6	 9f	 10f	118-120	78
7	 9g	 10g	123-125	80
8	 9h	 10h	110-114	84
9	 9i	 10i	154-156	81
10	 9j	 10j	104-106	86
11	 9k	 10k	130-132	85
12	 9l	 10l	157-159	85
13	 9m	 10m	124-126	86

Study of anticancer activity

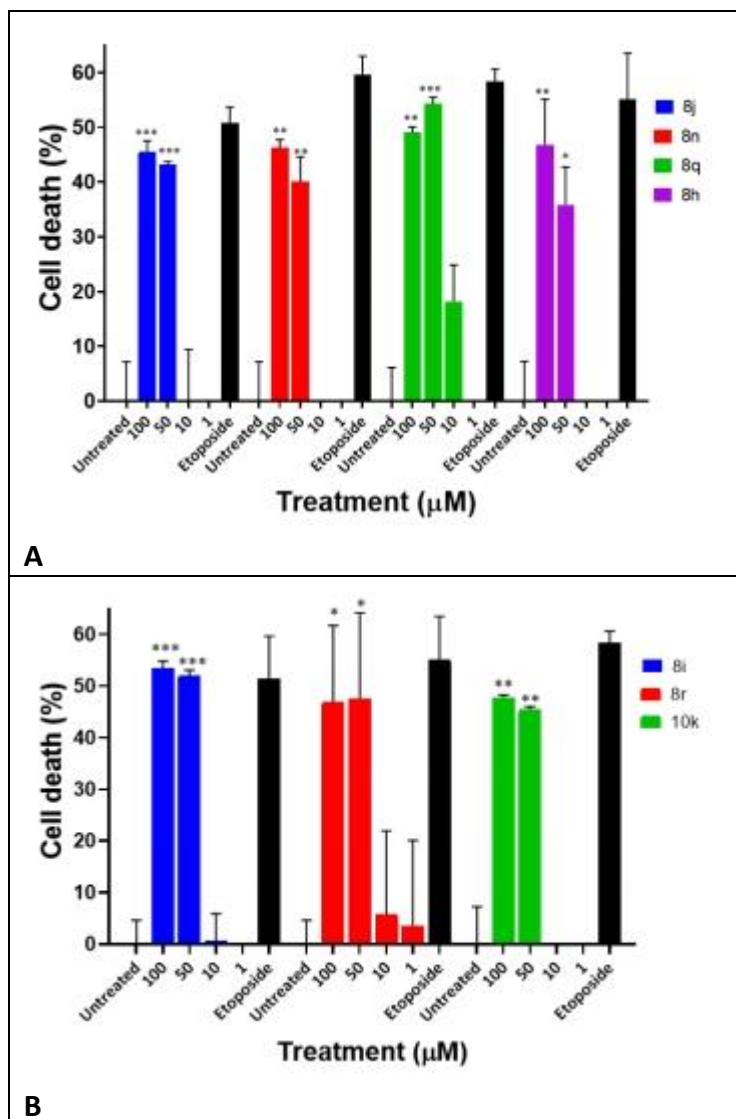


Figure 1. *In vitro* anticancer activity by MTT cell proliferation assay A) cytotoxicity of **8j**, **8n**, **8h** and **8q**. B) cytotoxicity of **8i**, **8r** and **10k**. Etoposide (10 μM) used as assay control. The data presented are the average of triplicate experiments and \pm SD (n = 3), statistical analysis performed two-tailed student's t test; * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Cytotoxicity. U-937 cells were used to study the cell death of novel synthesized compounds and the compounds **8j**, **8n**, **8h**, **8i**, **8q**, **8r** from Scheme I and **10k** from Scheme II have shown around 50% cell death at 50 μM concentration of each compound. The other compounds didn't show any significant cell death on U-937 cells. The cells treated with etoposide have shown around 50% of cell death at 10 μM. Among the effective compounds, **8j**, **8q** and **8i** were exhibited more significant anti-proliferative activity followed by **8n** and **10k** (Figure 1 A&B). The compounds **8h** and **8r** were slightly significant due to their experimental variations of % cell death. Popova *et.al.*²⁹ stated that tetrazole cycle is a capable pharmacophore widely used in the development of novel drugs. Over the decade, various isomeric forms of tetrazole have been advanced successfully for anticancer agents especially 5-oxo and 5-thiotetrazoles. In the present study, **8j**, **8n**, **8h**, **8i**, **8q**, **8r** and **10k** possessed a variety of substitutes on tetrazole cycle such as N-di(*o*-methoxy, *o*-fluoro), N-(*p*-

trifluorobenzyloxy), N-di(*p*-trifluorobenzyloxy), N-dibenzoyl, *o,m*-difluoro, *p*-methylbenzyloxy, N-(*o*-chloro, *p*-fluorobenzoyl) and *m,m,p*-trimethoxyphenyl respectively. Furthermore, the compounds **8j**, **8n**, **8h**, **8i**, **8q**, **8r** and **10k** characterizing towards anti-cancer mode of action.

Molecular docking study

Compound **1h** and **2k** were found to show better docking score among all the compound 1 and 2 series compounds when compared to control molecule Colchicine. Compounds **8h**, **10k**, and Colchicine had shown the docking score -157.142, -140.827, and -123.11 based on the free energy calculations used in the Molegro Virtual Docker. The compound **8h** in the tubulin active site (Figure 2) formed two hydrogen bonds with the ALA250 while **10k** (Figure 3) formed no hydrogen bonds. The compound **8h** and **10k** formed better interaction with the tubulin active site. Although Colchicine is known tubulin inhibitor, its docking score was comparatively less when compared to compound **8h** and **10k**. Both the compounds found to show better interaction with the tubulin than Colchicine. Among all the derivatives of 1 and 2 series, compound **8h** has shown highest binding interaction efficiency. The compound **8h** was found to be best interacting molecule in the tubulin active site among all the compound 1 and 2 derivatives.

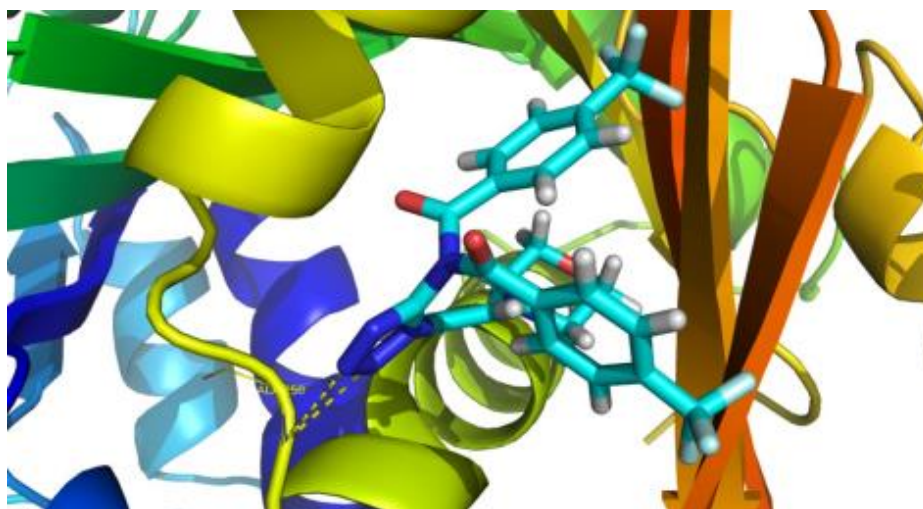


Figure 2. **8h** In tubulin binding site.

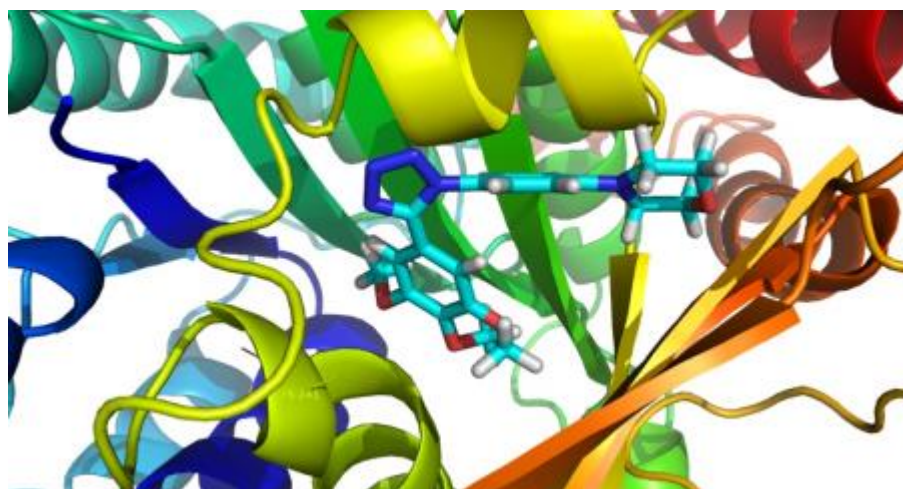


Figure 3. **10k** In tubulin binding site.

Conclusions

In conclusion, 3,4-difluoronitrobenzene was used as the starting material to generate tetrazole-morpholine hybrid derivatives. The crucial intermediate 4-(2-fluoro-4-(1*H*-tetrazol-1-yl)phenyl)morpholine (**5**) was developed, and it was then converted into two distinct tetrazole-morpholine linked hybrid series. To investigate the impact of various functional substitutions, including electron donating and withdrawing groups, a total of 31 compounds were synthesized. The anticancer activity of the synthesized compounds was evaluated in experiments using U-937 histiocytic lymphoma cells. Among all the drugs studied, **8h** and **10k** demonstrated the best interactions with tubulin when the mechanism of action was examined utilizing molecular docking against tubulin.

Experimental Section

General. Melting points were determined in open capillary tubes in sulphuric acid bath. FT-IR spectra were recorded on a VERTEX 70 Bruker by using KBr. A Bruker spectrometer 400 and 100 MHz was employed for recording ¹H NMR and ¹³C NMR spectra respectively and CDCl₃ and DMSO-*d*₆ were used as solvent and TMS as an internal standard. Mass spectra were recorded on Agilent-LCMS instrument.

Procedure for synthesis of 4-(2-fluoro-4-nitrophenyl)morpholine (3). To a stirred solution of 3,4-difluoronitrobenzene **1** (40 g, 251.4 mmol) in DMSO (400 mL) was added K₂CO₃ (44.4 g, 321.6 mmol) and morpholine **2** (24 g, 276.6 mmol) and heated to 80 °C for 3 h. The reaction mixture was cooled to room temperature and diluted with water (400 mL) and extracted with ethyl acetate (3x400 mL). The organic layer was separated and washed with brine solution (300 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to afford 4-(2-fluoro-4-nitrophenyl)morpholine **3** (48 g, Yield: 84%) as off white solid; mp: 112-114 °C; ¹H NMR (400 MHz, CDCl₃) δ= 8.01-7.93(m, 1H), 7.92 (d, *J* 2.8 Hz, 1H), 6.92 (t, *J* 8.8 Hz, 1H), 4.01 (t, *J* 4.8 Hz, 4H), 3.20 (t, *J* 4.8 Hz, 4H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ= 154.3, 151.8, 145.4, 120.9, 116.8, 112.6, 66.5 (2C), 49.8 (2C) ppm. IR (KBr): 3432, 2925, 1739, 1604, 1242, 1050 cm⁻¹. HRMS (ESI): calcd for C₁₀H₁₂N₂O₃F (M+H)⁺: 227.0832 found 227.0838.

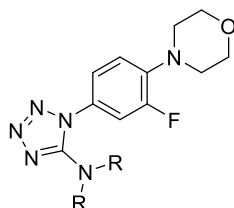
Procedure for synthesis of 3-fluoro-4-morpholinoaniline (4). To a stirred solution of 4-(2-fluoro-4-nitrophenyl)morpholine **3** (40 g, 177 mmol) in ethanol (360 mL) and water (40 mL) was added iron powder (94.16 g, 1681.47 mmol) and ammonium chloride (4.74 g, 88.48 mmol) and heated to 90 °C for 12 hours. The reaction mixture was cooled to room temperature and filtered through celite bed and washed with ethyl acetate, the organic layer was washed with water (400 mL) followed by brine (400 mL) solution. The organic layer was separated, dried over Na₂SO₄ and concentrated *in vacuo* to afford 3-fluoro-4-morpholinoaniline **4** as a pale brown solid (29.88g, Yield: 86%); mp: 125-127 °C; ¹H NMR (400 MHz, CDCl₃) δ= 6.81 (t, *J* 8.4 Hz, 1H), 6.45-6.39 (m, 2H), 3.85 (t, *J* 4.8 Hz, 4H), 3.54 (brs, 2H), 2.96 (t, *J* 4.8 Hz, 4H) ppm. ¹³C NMR (100 MHz, DMSO) δ = 157.47, 145.52, 129.03, 120.49, 109.52, 66.38, 51.60, 66.05, 50.20 ppm. HRMS (ESI): calcd for C₁₀H₁₄N₂O (M+H)⁺: 197.1090 found 197.1096.

Procedure for synthesis of 4-(2-fluoro-4-(1*H*-tetrazol-1-yl)phenyl)morpholine (5). To a stirred solution of 3-fluoro-4-morpholinoaniline **4** (20 g, 102.04 mmol) in acetic acid (100 mL) was added triethylorthoformate (24 g, 163.26 mmol) and NaN₃ (9.8 g, 153.06 mmol) and heated to 100 °C for 3 hours. The reaction mixture was cooled to room temperature and diluted with water (200 mL) and extracted with ethyl acetate (3x200 mL). The organic layer was washed with water (200 mL) followed by brine solution (150 mL), separated and dried

over Na₂SO₄ filtered and concentrated *in vacuo* to afford 4-(2-fluoro-4-(1*H*-tetrazol-1-yl)phenyl)morpholine **5** as off white solid (22.2 g, Yield: 87%); mp: 161-163 °C; ¹H NMR (400 MHz, CDCl₃) δ= 8.90 (s, 1H), 7.47-7.39 (m, 2H), 7.07 (m, 1H), 3.90 (t, *J* 6.4 Hz, 4H), 3.17 (t, *J* 6.4 Hz, 4H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ= 155.4, 142.1, 140.6, 127.5, 119.7, 117.6, 110.0, 65.9, 50.1 ppm. HRMS (ESI): calcd for C₁₁H₁₃N₅OF (M+H)⁺: 250.1104 found 250.1105.

Procedure for synthesis of 1-(3-fluoro-4-morpholinophenyl)-1*H*-tetrazol-5-amine (6). A stirred mixture of 4-(2-fluoro-4-(1*H*-tetrazol-1-yl)phenyl)morpholine **5** (16 g, 64.24 mmol), NaN₃ (6.2 g, 96.38 mmol), NaOH (3.8 g, 96.38 mmol) and Et₃N (12.8 g, 128.5 mmol) in *i*-PrOH (30 mL) was treated with DMSO (70 mL). The reaction mixture was stirred at room temperature until the gas evolution ceased (2 hours) and then was treated with glacial AcOH (11.4 g, 193.2 mmol). The resulting suspension was stirred at 90 °C for 2 hours. Cooled and diluted with water (200 mL). The precipitate was separated by filtration, washed with water and dried *in vacuo* at 50 °C to afford 1-(3-fluoro-4-morpholinophenyl)-1*H*-tetrazol-5-amine **6** as a white solid (14.0 g, Yield: 83%); mp: 207-209 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.27-7.24 (m, 2H), 7.07 (t, *J* 8.8 Hz, 1H), 4.80 (brs, 2H), 3.89 (t, *J* 4.8 Hz, 4H), 3.17 (t, *J* 4.8 Hz, 4H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ = 157.4, 155.0, 145.4, 129.0, 120.4, 109.5, 66.3 (2H), 51.5 (2H) ppm. IR (KBr): 3340, 3154, 2836, 1664, 1522, 1233, 1120cm⁻¹. HRMS (ESI): calcd for C₁₁H₁₄N₆OF (M+H)⁺: 265.1213 found 265.1218.

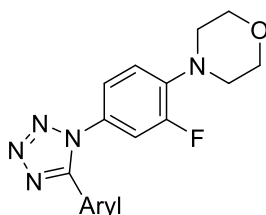
General procedure for compounds **8a-8j** (diacetylation tetrazole compounds)



1-(3-Fluoro-4-morpholinophenyl)-1*H*-tetrazol-5-amine **6** (150 mg, 0.56 mmol) was dissolved in THF (10 mL), cooled to 0 °C and added DIPEA (20 mg, 1.7 mmol) followed by acid chloride (**7a-7j**) (3 equiv., 1.7 mmol). The reaction mixture was stirred at room temperature for 12 h and quenched with water and extracted with ethyl acetate (3x10 mL). The combined organic layer was washed with brine solution (10 mL) and dried over Na₂SO₄ and concentration *in vacuo* to afford respective amide derivatives **8a-8j**. The analytical data of all compounds are given in the supplementary material.

General procedure for compound **8k-8r (Mono acetylation tetrazole compounds).** To a solution of 1-(3-fluoro-4-morpholinophenyl)-1*H*-tetrazol-5-amine **6** (150 mg, 0.56 mmol) was dissolved in THF (10 mL), cooled to 0 °C and added DIPEA (20 mg, 1.7 mmol) followed by acid chloride (**7k-7r**) (1.1 equiv., 0.616 mmol). The reaction mixture was stirred at room temperature for 2-6 hr and quenched with water and extracted with ethyl acetate (3x10 mL). The combined organic layer was washed with brine solution (10 mL) and dried over Na₂SO₄ and concentration *in vacuo* to afford respective amide derivatives **8k-8r**. The analytical data of all compounds are given in the supplementary material.

General procedure for compounds **10a-10m** (Aryl tetrazole compounds)



A suspension of tetrazole **5** (300 mg, 1.00 mmol), aryl iodide/Iodo benzene **9a-9m**(1.00 mmol), cesium carbonate (1.10 mmol), copper(I) iodide (1.00 mmol), palladium(II) acetate Pd(OAc)₂ (0.05 mmol), and tris(2-furyl)-phosphine (0.10 mmol) in dry acetonitrile (6 mL) was heated at 40 °C under argon atmosphere for 4 h. The resulting mixture was diluted with ethyl acetate (40 mL) and filtered quickly through celite, and then the solvents were removed under reduced pressure. The residue was purified by column chromatography in hexane and ethyl acetate to afford tetrazole derivatives (**10a-10m**) (392 mg, 87%). The analytical data of all compounds are given in the supplementary material.

Study of anticancer activity

Cytotoxicity assay. To reveal anticancer activities of compounds under *in vitro* conditions, MTT- cell proliferation assay was performed³⁰. U-937 cells (5 X 10³ /well) seeded in 96-well plate were incubated at 37 °C with 5% CO₂ for 45 min prior to synthesised compound treatment. Then, cells were treated with compound for 48 hours in triplicates and cells treated with 10 µM etoposide were taken as positive control for the assay. Following the incubation, 10 µl of MTT dye [(3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide), stock 4mg/ml in PBS] was added and cells were incubated at 37 °C with 5% CO₂ for 3 hours in dark. Then, MTT lysis buffer containing 20% SDS and 50% DMF in 1:1 ratio was added to each well and kept overnight on orbital shaker at room temperature in dark. The following day, the absorbance of each well was recorded at 595 nm in BIO-RAD iMark Microplate Reader and data was visualized using Microplate Analyst software. The average cell death was calculated and standard deviation was shown as error bar. The experiments were performed in triplicates.

Statistical analysis. For statistical analysis, a two-tailed t test (paired) was performed. Between the treated and untreated sets of experiments. A p value of < 0.05 is reflected to considered slightly significant and specified with an asterisk (*), a p value < 0.01 is significant with a double asterisk (**), and a p value < 0.001 is considered as more significant and marked with triple asterisk (***)

Methods. Docking study

The X-ray crystal structure 1SA0 of the tubulin was retrieved from the protein structural database protein data bank (PDB). Derivatives of compound 1 and 2 were sketched in marvinsketch. Molecular docking was performed between tubulin and compound 1 and 2 derivatives to validate the binding efficiency based on the molecular docking scores. The synthesized compounds were docked on to the tubulin protein active site with Molegro Virtual Docker 6.0 tool. The known tubulin inhibitor Colchicine was used as reference molecule for docking score comparison. Active site coordinates 116.99, 90.54, and 5.65 with 15 Å radius was considered for docking. Docked ligand poses in active site were analyzed in Pymol protein visualization tool.

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Supplementary Material

Spectra of compounds **3, 4, 5, 6, 8a-8r, 10a-m**

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