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Novel 5-Methyl-2-[(un)substituted phenyl]-4-{4,5-dihydro-3-[(un)substituted phenyl]-5-(1,2,3,4-tetrahydroisoquinoline-2-yl)pyrazol-1-yl}-oxazole Derivatives: Synthesis and Anticancer Activity

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Eleven novel 5-methyl-2-[(un)substituted phenyl]-4-{4,5-dihydro-3-[(un)substituted phenyl]-5-(1,2,3,4-tetrahydroisoquinoline-2-yl)pyrazol-1-yl}-oxazole derivatives were synthesized and characterized by elemental analysis, ESI-MS, ^{1}H NMR and ^{13}C NMR. All of the compounds have been screened for their antiproliferative activities against PC-3 cell (human prostate cancer) and A431 cell (human epidermoid carcinoma cancer) lines *in vitro*. The results revealed that compounds **4g**, **4j** and **4k** exhibited the strong inhibitory activities against the PC-3 cell lines (with IC_{50} values of 2.8±0.11, 3.1±0.10 and 3.0±0.06 μg/mL, respectively).

Keywords synthesis, aryl-dihydropyrazole, 1,2,3,4-tetrahydroisoquinoline, anticancer activity

Introduction

Oxazoles are key building elements of natural products. Among the numerous heterocyclic moieties of biological and pharmacological interest, the oxazole ring is endowed with various activities,[1,2] and has gained considerable attention in the last ten years.[3-5] The substituted oxazole system occurs in various antivirals and antibiotics. For example, sulfomycin I, a novel thiopeptide antibiotic produced by a subspecies of *Steptomyces viridochromogenes*, exhibits strong bacterium inhibitory activity. The oxazole ring is most commonly obtained by the Hantzsch reaction[6] or the cyclodehydration of *β*-ketoamides,[7] alike the dehydrogenation of oxazolines and other processes such as Schmidt rearrangements[8] have also been employed. Heterocyclic compounds incorporating an oxazole moiety exhibit a wide range of bioactivities, *e.g*. antitumor growth inhibition in human colorectal DLD-1 xenograft mouse model,[9] anticancer activity against human prostate and epidermoid carcinoma cells.[10,11] Tai[9] has reported the *in vivo* activities and their structure-activity relationships (SAR) of a new class of 2-aryl-oxazole-4-carboxamides. Furthermore, incorporation of fluorine into aromatic and heterocyclic moieties is known to significantly enhance the anticancer activity of the molecule. Fascinated by these findings and our own results,[10-12] with an aim to develop new and potent inhibitors with anticancer activity, herein we designed a series of novel isoquinoline-pyrazole-methyloxazole derivatives containing fluorinated functionality. The synthetic route to target compounds is shown in Scheme 1. The structures of novel compounds were confirmed by spectral analysis. The compounds were evaluated for their antiproliferative activities against PC-3 and A431 cell lines *in vitro* by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) method.

Results and discussion

The preparation of the intermediate *α*, *β* unsaturated ketone **1** is the key step for the synthesis of title compounds. Nicolaou *et al*.[13] have demonstrated that HIO_3 and its anhydride I_2O_5 were the mild and selective alternative reagents for the dehydrogenation of aldehyde and ketone. Using this method, from control reactions under a range of thermal conditions, *α*,*β*-unsaturated ketone **1** was successfully prepared from 2-(2-benzoylethyl)-1,2,3,4-tetrahydroisoquinoline. Compounds **2** and **3** (Scheme 1) were synthesized according to the previously published reports.[14]

Microwave chemistry, a non conventional popular

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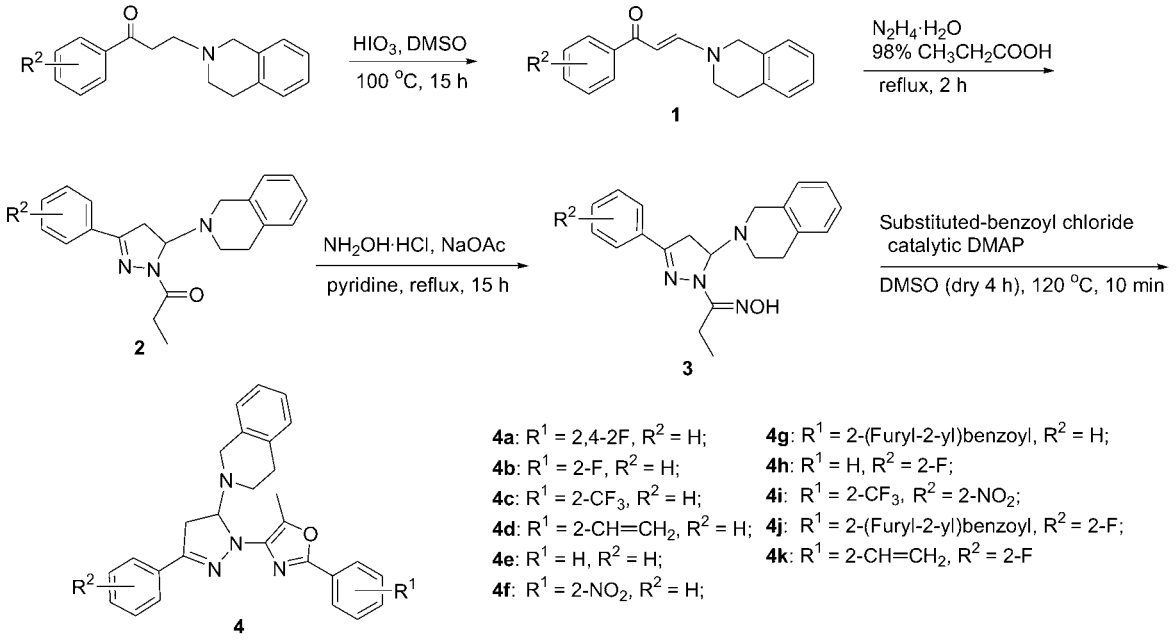
Scheme 1 Synthesis of 5-methyl-2-[(un)substituted phenyl]-4-{4,5-dihydro-3-[(un)substituted phenyl]-5-(1,2,3,4-tetrahydroisoquinoline-2-yl)pyrazol-1-yl}-oxazole



technique, has been successfully employed in the preparation of oxazole. Lee *et al.*[15] developed a solvent free method in which (hydroxy-(2,4-dinitrobenzenesulfonyloxy)iodo)benzene reacts with various ketones in a household microwave to form *α*-((2,4-dinitrobenzene)-sulfonyl)oxy ketones, which are converted to oxazoles through treatment with amides. Microwave irradiation is also known to promote the rapid *O*,*N*-acylation-cyclodehydration cascade reaction of oximes and acid chlorides to give oxazoles.[16] Malamas *et al.*'s method[17] for converting oxime to oxazole under microwave irradiation at various temperatures in pyridine/toluene (5.6∶1, molar ratio) actually turned out to be a failure in our hands. Then catalytic DMAP in DMF was added in to attempt to facilitate acyl transferring processes, and with this optimal condition, compounds **4a**—**4k** were prepared.

In vitro anticancer assay

In the screening assay studies, all the compounds were evaluated for their cytotoxic activity against two cell lines, PC-3 and A431. The cells were allowed to proliferate in the presence of tested material for 48 h, and the results are reported in terms of IC_{50} values (Table 1). From the IC_{50} values, it is obvious that compounds **4d**, **4g**, **4h**, **4j** and **4k** exhibited the strong inhibitory activity (with IC_{50} of 3.5±0.05, 2.8±0.11, 3.9±0.18, 3.1±0.10 and 3.0±0.06 μg/mL, respectively) against the PC-3 cell lines and the values could compare with that of the potent 5-fluorouracil serving as a positive control. All the compounds showed poor activity against A431 cell lines, which is comparable to that of the positive control.

The rationale behind selecting a large number of compounds bearing different functionalities was to establish a definite structure-activity relationship pattern and emphasize the role of fluorine in imparting bioactivity. The choice of compounds was also governed by availability of the reagents and ease of preparation of title compounds under laboratory conditions. Scanning Table 1, we found that there was clear SAR against PC-3 cell lines. Inspection of the chemical structures of the final compounds (Scheme 1) suggests that the nature of group R^2 in the title compounds significantly influ-

Table 1 Cytotoxic activity of the synthesized compounds against PC-3 and A431cell lines[a]

Compound	IC_{50}[b]/(μg•mL^{-1})		Compound	IC_{50}[b]/(μg•mL^{-1})	
	PC-3	A431		PC-3	A431
4a	4.6±0.13	85.4±0.21	**4d**	3.5±0.05	74.8±0.11
4b	6.3±0.06	70.9±0.07	**4e**	7.0±0.06	81.3±0.05
4c	5.8±0.11	120.3±0.11	**4f**	8.9±0.22	98.9±0.13
4h	3.9±0.18	100.3±0.04	**4g**	2.8±0.11	69.7±0.18
4i	20.3±0.11	64.1±0.02	**4j**	3.1±0.10	86.6±0.10
5-Fluorouracil[c]	2.2±0.12	2.1±0.20	**4k**	3.0±0.06	90.8±0.15

[a] The data represented the mean of three experiments in triplicate and were expressed as mean±SD. [b] The IC_{50} value was defined as the concentration at which 50% survival of cells was observed. The results are listed in the table. [c] Used as a positive control.

ence the antitumor activity. With a fluorinated substituent (2-F) on the phenyl ring, the compounds exhibited enhanced bioactivity against PC-3 cell lines (**4h**, **4j**, **4k**). Furthermore, the presence of a heterocycle-functional group in the title compounds [2-(furyl-2-yl)benzoyl] plays an important role in the antiproliferative activity (**4g** and **4j**). In addition to compound **4i**, all the synthetic compounds showed almost the same antiproliferative activity against PC-3 cell lines. In summary, some 5-methyl-2-[(un)substituted phenyl]-4-{4,5-dihydro-3-[(un)substituted phenyl]-5-(1,2,3,4-tetrahydroisoquinoline-2-yl)pyrazol-1-yl}-oxazole derivatives have potentially high antiproliferative activity against PC-3 cell lines and deserve further investigation.

Experimental

General

Melting points were measured and not corrected. ^{1}H NMR spectra were recorded on a Varian INOVA300 (500 MHz) pulse Fourier-transform NMR spectrometer in $CDCl_3$. ^{13}C NMR spectra were recorded on a Varian INOVA400 (125 MHz) pulse Fourier-transform NMR spectrometer. ESI mass spectra were obtained on a Mariner System 5303 mass spectrometer. Elemental analysis were performed by a Vario-III CHN analyzer and were within ±0.4% of the theoretical values. The reagents employed were of analytical grade. Compound **1** was prepared according to a literature method as described.[13]

Synthesis

General synthetic procedure for 5-methyl-2-[(un)substituted phenyl]-4-{4,5-dihydro-3-[(un)substituted-phenyl]-5-(1,2,3,4-tetrahydroisoquinoline-2-yl)pyrazol-1-yl}-oxazole (4) To a solution of oxime **3** (2 mmoL) and *N*-methylmorpholine (0.010 mmoL) in DMF (30 mL) in 0—10 ℃ was added dropwise acid chloride (3.0 mmol) for 30 min. The reaction mixture was heated in the microwave for 10 min at 120 ℃ and poured into water (50 mL), then the solution was maintained in 0—5 ℃ for 10 h. The product was collected by filtration, and the crude residue was purified by chromatography on SiO_2 (acetone∶petroleum, V∶V=2∶1) to give **4** as a colorless solid. Their spectra were given in the supporting information.

4a ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.37 (s, 3H, Me), 2.50 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.62 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.77 (t, J=3.7 Hz, 2H, isoquinoline-H), 2.82 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.69 (s, 2H, isoquinoline-H), 4.50 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 6.78—7.92 (m, 12H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.6, 27.7, 37.9, 47.2, 56.6, 69.2, 105.5, 111.7, 120.2, 125.9, 126.4, 127.5, 127.8, 129.1, 129.3, 129.5, 130.2, 132.0, 133.6, 135.1, 138.2, 138.4, 152.5, 159.7, 160.8, 165.6; ESI-MS m/z: 469.3 ($C_{28}H_{24}F_2N_4O$, $[M+H]^+$). Anal. calcd for $C_{28}H_{24}F_2N_4O$: C 71.48, H 5.14, N 11.91; found C 71.69, H 5.33, N 12.38.

4b ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.38 (s, 3H, Me), 2.54 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.65 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.77 (t, J=3.7 Hz, 2H, isoquinoline-H), 2.84 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.65 (s, 2H, isoquinoline-H), 4.45 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 6.99—7.81 (m, 13H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.2, 28.0, 38.3, 47.6, 56.4, 69.0, 116.2, 124.7, 125.2, 125.7, 127.4, 127.9, 128.7, 129.1, 129.4, 129.6, 129.7, 130.5, 131.3, 133.6, 134.7, 138.0, 139.0, 152.4, 159.1, 160.6; ESI-MS m/z: 452.0 ($C_{28}H_{25}FN_4O$, $[M+H]^+$). Anal. calcd for $C_{28}H_{25}FN_4O$: C 74.32, H 5.57, N 12.38; found C 74.00, H, 5.80, N 12.11.

4c ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.35 (s, 3H, Me), 2.50 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.61 (t, J=3.4 Hz, 2H, isoquinoline-H), 2.78 (t, J=3.7 Hz, 2H, isoquinoline-H), 2.87 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.69 (s, 2H, isoquinoline-H), 4.51 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 7.03—7.86 (m, 13H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.5, 27.8, 38.3, 47.6, 56.6, 68.8, 118.1, 125.7, 125.9, 126.0, 127.3, 127.5, 128.1, 128.3, 128.8, 129.1, 129.9, 130.1, 130.8, 132.0, 133.7, 133.8, 137.7, 139.2, 152.5, 159.1, 160.4; ESI-MS m/z: 503.1 ($C_{29}H_{25}F_3N_4O$, $[M+H]^+$). Anal. calcd for $C_{29}H_{25}F_3N_4O$: C 69.31, H 5.01, N 11.15; found C 69.01, H 5.39, N 11.41.

4d ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.32 (s, 3H, Me), 2.60 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.72 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.80 (t, J=3.7 Hz, 2H, isoquinoline-H), 2.91 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.75 (s, 2H, isoquinoline-H), 4.57 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 5.50 (dd, J=10.5, 1.3 Hz, 2H, =CH_2), 6.99 (dd, J=17.2, 1.3 Hz, 1H, =CH), 7.02—7.81 (m, 13H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.6, 28.0, 38.0, 47.4, 56.3, 69.1, 114.8, 125.8, 126.1, 127.0, 127.6, 127.9, 128.1, 128.8, 129.0, 129.3, 129.5, 130.0, 132.1, 133.7, 134.5, 134.8, 135.1, 137.9, 138.3, 139.0, 152.4, 160.8; ESI-MS m/z: 460.1 ($C_{30}H_{28}N_4O$, $[M+H]^+$). Anal. calcd for $C_{30}H_{28}N_4O$: C 78.23, H 6.13, N 12.16; found C 78.00, H 6.47, N 12.01.

4e ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.33 (s, 3H, Me), 2.51 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.67 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.78 (t, J=3.6 Hz, 2H, isoquinoline-H), 2.88 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.67 (s, 2H, isoquinoline-H), 4.52 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 7.04—7.88 (m, 14H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.5, 28.0, 38.1, 47.8, 56.5, 69.2, 126.1, 126.5, 126.8, 127.6, 127.9, 128.2, 128.6, 128.9, 129.2, 129.3, 129.5, 132.0, 134.2, 135.1, 138.0, 138.9, 152.6, 160.4; ESI-MS m/z: 433.6 ($C_{28}H_{26}N_4O$, $[M+H]^+$). Anal. calcd for $C_{28}H_{26}N_4O$: C 77.39, H 6.03, N 12.89l; found C 77.02, H 6.35, N 13.07.

4f ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.35 (s, 3H, Me), 2.50 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.66 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.78 (t, J=3.7 Hz, 2H, isoquinoline-H), 2.84 (dd, J=18.0, 11.0 Hz, pyrazole,

1H, 4-H_b), 3.69 (s, 2H, isoquinoline-H), 4.55 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 7.00—8.36 (m, 13H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.3, 27.7, 38.5, 48.2, 56.3, 69.0, 122.3, 126.0, 126.3, 127.7, 128.2, 128.8, 129.0, 129.1, 129.9, 130.3, 132.0, 132.3, 134.0, 134.8, 135.7, 138.0, 139.1, 147.2, 152.6, 160.6; ESI-MS *m*/*z*: 480.1 ($C_{28}H_{25}N_5O_3$, $[M+H]^+$). Anal. calcd for $C_{28}H_{25}N_5O_3$: C 70.13, H 5.25, N 14.60; found C 70.08, H 5.47, N 14.21.

4g ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.38 (s, 3H, Me), 2.52 (dd, J=18.0, 3.1 Hz, 1H, pyrazole, 4-H_a), 2.69 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.84 (t, J=3.7 Hz, 2H, isoquinoline-H), 3.05 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.79 (s, 2H, isoquinoline-H), 4.58 (dd, J=11.0, 3.1 Hz, 1H, pyrazole, 5-H), 6.37 (d, J=3.3 Hz, 1H, furyl), 6.55 (d, J=3.4 Hz, 1H, furyl), 7.00—7.86 (m, 14H, furyl 1H and 10ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 4.9, 27.7, 38.2, 48.1, 56.2, 68.8, 106.7, 109.3, 126.0, 126.2, 127.7, 128.0, 128.7, 129.1, 129.2, 129.5, 129.7, 130.1, 130.2, 132.0, 133.9, 134.8, 136.3, 136.5, 138.0, 139.1, 144.3, 152.5, 155.0, 160.1; ESI-MS *m*/*z*: 498.9 ($C_{32}H_{28}N_4O_2$, $[M+H]^+$). Anal. calcd for $C_{32}H_{28}N_4O_2$: C 76.78, H 5.64, N 11.19; found C 77.00, H 5.40, N 11.55.

4h ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.32 (s, 3H, Me), 2.55 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.67 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.72 (t, J=3.6 Hz, 2H, isoquinoline-H), 2.80 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.62 (s, 2H, isoquinoline-H), 4.51 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 7.02—7.87 (m, 13H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.0, 28.2, 38.5, 47.9, 56.7, 69.0, 116.5, 120.0, 124.1, 126.0, 126.3, 127.0, 127.2, 127.9, 128.0, 128.3, 128.9, 130.0, 130.7, 133.0, 134.5, 138.0, 138.9, 152.7, 160.3, 160.8; ESI-MS *m*/*z*: 451.7 ($C_{28}H_{25}FN_4O$, $[M+H]^+$). Anal. calcd for $C_{28}H_{25}FN_4O$: C 74.32, H 5.57, N 12.38; found C 74.60, H 5.25, N 12.79.

4i ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.37 (s, 3H, Me), 2.54 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.66 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.73 (t, J=3.6 Hz, 2H, isoquinoline-H), 2.80 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.69 (s, 2H, isoquinoline-H), 4.51 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 6.97—8.41 (m, 12H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.5, 28.2, 38.0, 48.5, 56.5, 69.4, 119.0, 123.0, 125.6, 125.9, 126.0, 126.2, 127.7, 128.1, 128.7, 129.0, 130.3, 130.6, 131.2, 131.9, 132.3, 132.6, 134.1, 135.2, 138.2, 139.1, 147.8, 152.3, 160.2; ESI-MS *m*/*z*: 548.0 ($C_{29}H_{24}F_3N_5O_3$, $[M+H]^+$). Anal. calcd for $C_{29}H_{24}F_3N_5O_3$: C 63.62, H 4.42, N 12.79; found C 64.05, H 4.87, N 12.91.

4j ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.36 (s, 3H, Me), 2.50 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.67 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.86 (t, J=3.7 Hz, 2H, isoquinoline-H), 3.00 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.73 (s, 2H, isoquinoline-H), 4.51 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 6.42 (d, J=3.3 Hz, 1H, furyl), 6.58 (d, J=3.4 Hz, 1H, furyl), 7.02—7.79 (m, 13H, furyl 1H and 10ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.5, 28.3, 38.5, 48.4, 56.1, 69.4, 106.2, 107.9, 116.1, 119.3, 124.4, 126.1, 126.3, 127.9, 128.3, 128.5, 128.7, 129.1, 129.8, 130.2, 131.2, 132.4, 134.5, 136.1, 136.7, 138.0, 139.1, 143.3, 152.7, 155.6, 160.3, 160.9; ESI-MS *m*/*z*: 517.9 ($C_{32}H_{27}FN_4O_2$, $[M+H]^+$). Anal. calcd for $C_{32}H_{27}FN_4O_2$: C 74.11, H 5.25, N 10.80; found C 74.38, H 4.89, N 10.52.

4k ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.38 (s, 3H, Me), 2.54 (dd, J=18.0, 3.0 Hz, 1H, pyrazole, 4-H_a), 2.70 (t, J=3.5 Hz, 2H, isoquinoline-H), 2.84 (t, 2H, J=3.7 Hz, isoquinoline-H), 2.96 (dd, J=18.0, 11.0 Hz, pyrazole, 1H, 4-H_b), 3.71 (s, 2H, isoquinoline-H), 4.51 (dd, J=11.0, 3.0 Hz, 1H, pyrazole, 5-H), 5.53 (dd, J=10.5, 1.3 Hz, 2H, $=CH_2$), 6.93 (dd, J=17.1, 1.3 Hz, 1H, $=CH$), 7.02—7.75 (m, 12H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 5.3, 28.0, 38.4, 48.1, 56.1, 69.0, 114.2, 116.1, 119.0, 124.2, 125.9, 126.3, 127.4, 127.6, 128.0, 128.5, 128.7, 129.0, 129.7, 131.2, 133.3, 134.7, 135.3, 135.5, 137.9, 138.2, 139.1, 152.2, 160.3, 161.1; ESI-MS *m*/*z*: 479.2 ($C_{30}H_{27}FN_4O$, $[M+H]^+$). Anal. calcd for $C_{30}H_{27}FN_4O$: C 75.29, H 5.69, N 11.71; found C 75.00, H 6.04, N 11.88.

Bioassay conditions

The cytotoxicity evaluation was conducted by using a modified procedure as described in the literature. Briefly, target tumor cells were grown to log phase in RPMI 1640 medium supplemented with 10% fetal bovine serum. After diluting to 3×10^4 cells/mL with the complete medium, 100 μL of the obtained cell suspension was added to each well of 96-well culture plates. The subsequent incubation was performed at 37 ℃, 5% CO_2 atmosphere for 24 h before subjecting the suspension to cytotoxicity assessment. Tested samples at pre-set concentrations were added to 6 wells with 5-fluorouracil co-assayed as a positive reference. After a 48 h exposure period, 25 μL of PBS containing 2.5 mg/mL of MTT was added to each well. After 4 h, the medium was replaced by 150 μL of DMSO to dissolve the purple formazan crystals produced. The absorbance at 570 nm of each well was measured on an ELISA plate reader. The data represented the mean of three experiments in triplicate and were expressed as mean±SD using *Student t* test. The IC_{50} value was defined as the concentration at which 50% of the cells could survive.

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