

Full Paper

1-Acetyl-3,5-diaryl-4,5-dihydro(1*H*)pyrazoles: Exhibiting Anticancer Activity through Intracellular ROS Scavenging and the Mitochondria-Dependent Death Pathway

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A series of 17 analogs of 1-acetyl-4,5-dihydro(1*H*)pyrazoles (**JP-1** to **JP-17**) bearing two aromatic rings at positions 3 and 5, either of which ought to be heterocyclic, were synthesized and evaluated for their anti-proliferative potential against breast cancer (MCF-7 and T-47D) and lung cancer (H-460 and A-549) cell lines for the first time. **JP-1-7**, **-10**, **-11**, **-14**, and **-15** were observed to exhibit significant anti-proliferative activity against MCF-7 cells. Some notions about structure–activity relationships are reported. The investigated compounds were found to lower the intracellular reactive oxygen species in the H₂DCFDA assay and also caused mitochondria-dependent cell death in the MCF-7 cell line, indicating a plausible mechanism of their anticancer effect.

Keywords: Antiproliferative / MCF cell lines / Mitochondria-dependent cell death / Pyrazoles / Synthesis

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Introduction

Cancer can be defined as the abnormal growth of cells resulting from multiple changes in gene expression, consequently disturbing the balance between cell proliferation and cell death and culminating in significant morbidity and death if untreated [1–3]. Over the past few decades numerous compounds have been synthesized, many of which, having subsequently qualified clinical trials and testing, are eventually accredited as efficacious chemotherapeutic agents [4–10]. Many of these chemotherapeutic agents, for their anticancer activity, are indebted to the heterocyclic scaffold [11–13] such as pyrazole [14], indole, quinazoline, imidazoline, pyridine, etc. Heterocycles have been known to greatly increase solubility in water or to bring about the reversible distortion

to the membrane structure [15, 16]. Heterocycles also possess the ability to alter drug properties such as potency, selectivity, lipophilicity, polarity, and aqueous solubility, thus playing an extremely crucial role in the design of therapeutic molecules [13]. Recently chalcones have gained considerable attention as they act not only as precursors for the synthesis of various derivatives, including pyrazoles, but also owing to the fact that they themselves exhibit broad spectrum of biological activities including anticancer [17–20]. Further, it was observed that pyrazoles when incorporated across the double bonds of chalcones [21] and combretastatins [22], resulted in better cytotoxic activity. In addition, acetylating the *N*1 of the pyrazole was observed to further enhance anticancer activity [23]. This ability could be attributed to the twisted structure or the angular conformation that pyrazolines could adopt, as opposed to pyrazoles, which possess a planar conformation, which showed significant interaction with the biological target [24, 25].

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Keeping in view of the above revelations, we thought of designing *N*¹-acetyl pyrazolines linked with two aromatic moieties of which at least one is heteroaromatic (**JP-1-17**; Fig. 1).

Results and discussion

Synthesis

All the final compounds (**JP-1-17**) were synthesized via a two-step process. Briefly, a heteroaryl/aryl ketone (**1**) and an aldehyde (**2**) were allowed to undergo base catalyzed Claisen–Schmidt condensation [26] to afford chalcones **JA-1-17** (yield 75–88%). The subsequent step involved Michael condensation of hydrazine hydrate with chalcones followed by cyclization (via imine bond formation) and *N*-acetylation in the presence of acetic acid under reflux to give 1-acetyl-3,5-diaryl-4,5-dihydro(1*H*)-pyrazoles (**JP-1-17**; Scheme 1). The target compounds were purified through recrystallization from methanol. All the target compounds (Table 1) were characterized by m.p., IR, NMR, and HRMS. The ¹H NMR of pyrazolines obtained showed typical ABX system characterized by three double doublets, out of which one double doublet was sufficiently deshielded and appeared more downfield than the other two.

Antiproliferative activity

In order to test the cytotoxic potential of the synthesized compounds, *in vitro* cytotoxic assay (MTT assay) was performed for the synthesized pyrazoline derivatives (**JP-1-17**) against breast (MCF-7 and T-47D) and lung (A-549 and H-460) cancer cell lines. Approximately 8000–10,000 cells were seeded per well of the 96-well plate and treatments with indicated drug concentrations were given in triplicate for 48 h followed by MTT assay. Results were expressed as inhibitory potential possessed by the synthesized compounds. Etoposide was used as standard anticancer agent [27]. The synthesized target compounds when tested on peripheral human blood lymphocytes were found to be non-toxic indicating that compounds had no effect on the normal cells. Although the synthesized pyrazoline derivatives were found to be devoid of activity against the lung cancer cell lines A-549 and H-460, they exhibited potent activity against MCF-7 (Fig. 2) cell lines and only to a small extent in T-47D (Fig. 3). The results of the IC₅₀ values, of some of the potential compounds, thus obtained are summarized in Table 2. The potency against the MCF-7 cell line indicates their effectiveness against non-metastatic hormonal dependent breast cancer cells while the higher grade cancer cell line seemed less sensitive toward the treatments. The unresponsiveness of the lung cancer

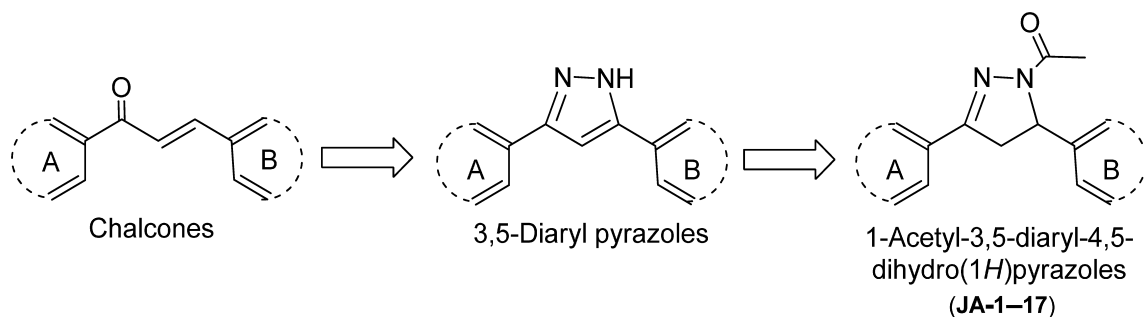
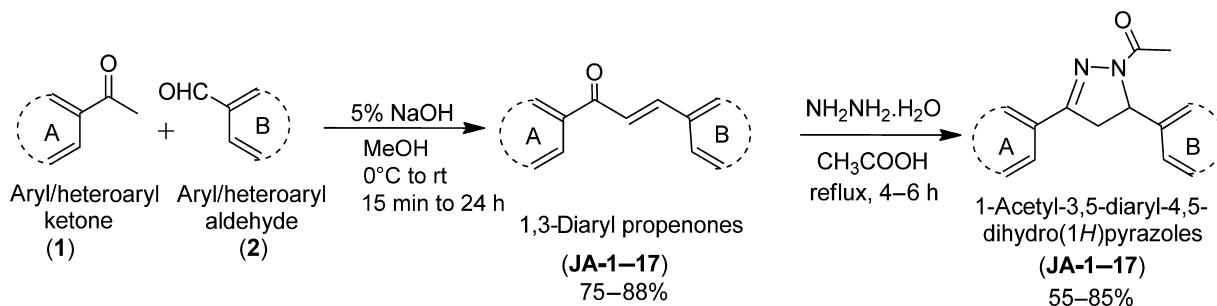
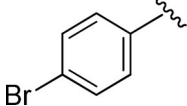
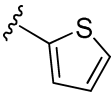
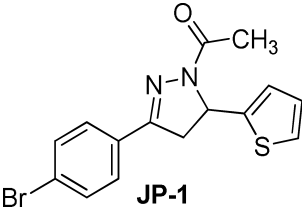
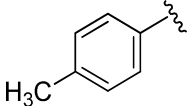
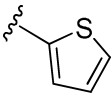
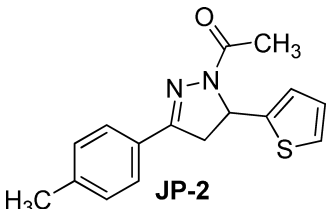
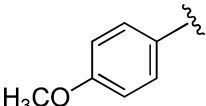
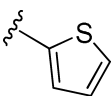
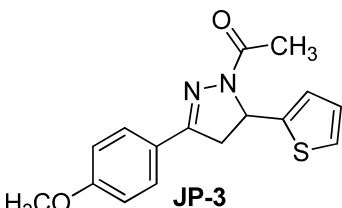
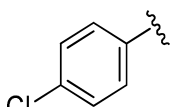
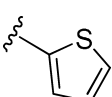
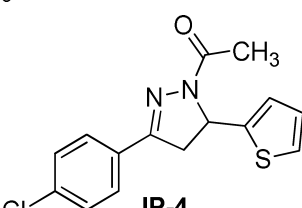
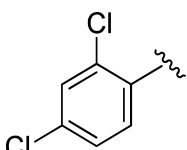
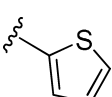
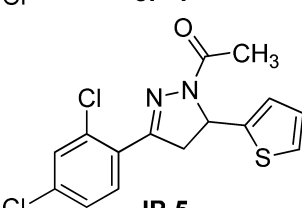
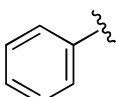
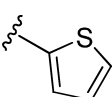
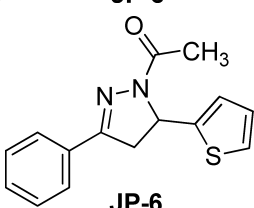
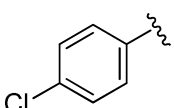
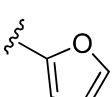
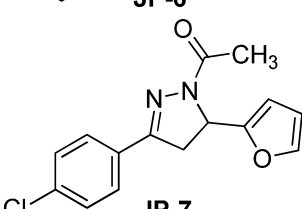


Figure 1. Design of the target compounds (**JP-1-17**)



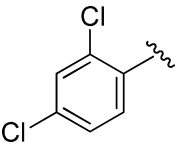
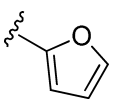
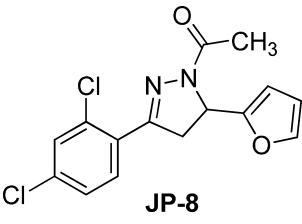
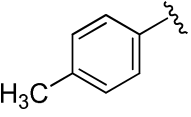
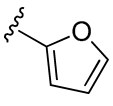
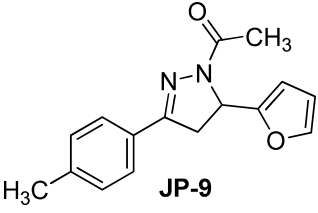
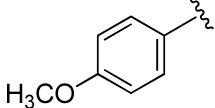
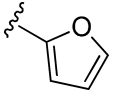
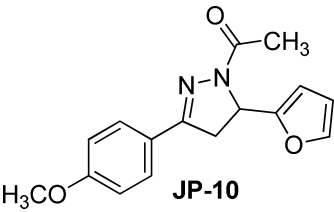
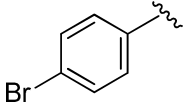
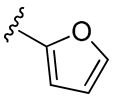
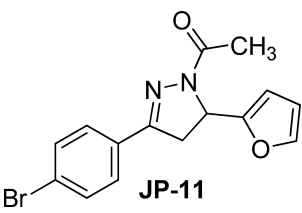
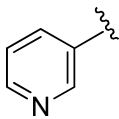
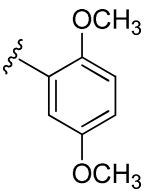
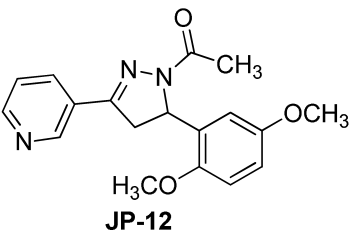
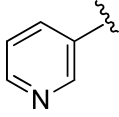
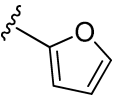
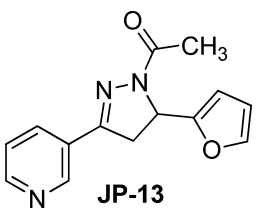
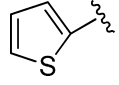
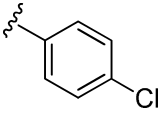
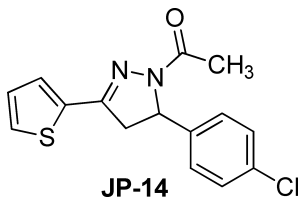
Scheme 1. Synthesis of the target compounds.

Table 1. Chemical structures of target compounds.

Entry	Ring A	Ring B	Synthesized compounds	Yield (%) ^{a)}
1			 JP-1	65
2			 JP-2	55
3			 JP-3	56
4			 JP-4	67
5			 JP-5	85
6			 JP-6	61
7			 JP-7	72

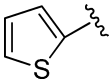
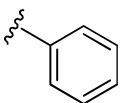
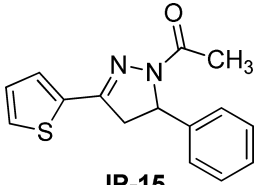
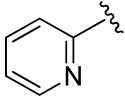
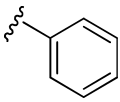
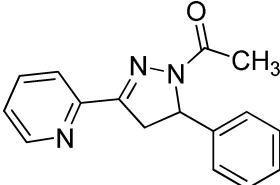
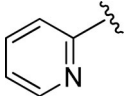
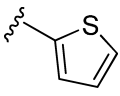
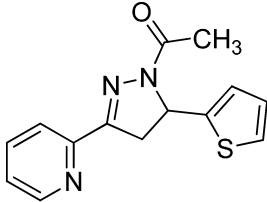
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Table 1. (Continued)

Entry	Ring A	Ring B	Synthesized compounds	Yield (%) ^{a)}
8			 JP-8	64
9			 JP-9	70
10			 JP-10	59
11			 JP-11	68
12			 JP-12	74
13			 JP-13	63
14			 JP-14	77

(Continued)

Table 1. (Continued)

Entry	Ring A	Ring B	Synthesized compounds	Yield (%) ^{a)}
15			 JP-15	56
16			 JP-16	60
17			 JP-17	55

^{a)} Isolated yield.

cell line highlights the enhanced tumorigenicity and self-renewal capacity of these cell lines [28].

MCF-7 cells exhibited significant sensitivity toward the synthesized compounds especially **JP-1**, **-5**, **-7**, and **-14**, which displayed potent inhibitory potential at low concentration of 5 μ M. At the same concentration, moderate inhibitory potential was observed for **JP-5** and **-7** whereas **JP-3**, **-4**, **-10**, and **-11** showed very low inhibitory potential. At 25 μ M, **JP-1**,

-5, **-7**, **-14**, and **-15** exhibited promising inhibitory potential as compared to **JP-2**, **-3**, **-10**, and **-11**, which showed comparatively little inhibitory potential. At higher concentration of 50 μ M, **JP-1**, **-5**, **-7**, and **-14** portrayed significant inhibitory potential with **JP-2**, **-3**, **-10**, and **-11** showing mild activity whereas some of the other synthesized compounds either showed negligible activity or were completely devoid of activity. An attempt to assess the anti-proliferative potential of the target compounds

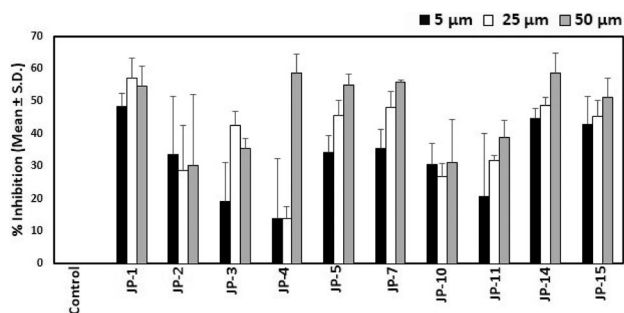


Figure 2. Percent inhibition of MCF-7 cells in response to treatment with synthesized compounds at concentrations of 5, 25, and 50 μ M for a time duration of 48 h. Data are expressed as mean values $\pm$ SD of three independent experiments.

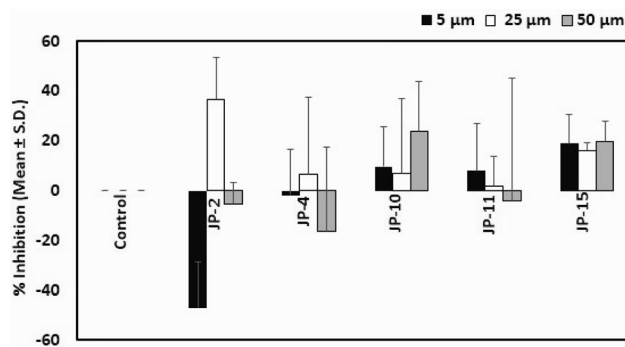


Figure 3. Percent inhibition of T-47D cells in response to treatment with synthesized compounds at concentrations of 5, 25, and 50 μ M for a time duration of 48 h. Data are expressed as mean values $\pm$ SD of three independent experiments.

Table 2. IC₅₀ of synthesized compounds.

Compound	IC ₅₀ (μM)	
	MCF-7	T-47D
JP-1	7	NT
JP-2	>50	>100
JP-3	>50	>100
JP-4	48	>100
JP-5	30	NT
JP-7	28	NT
JP-10	>50	>100
JP-11	>50	>100
JP-14	24	NT
JP-15	49	>100
Etoposide	20.9 [27]	ND

NT, not tested due to solubility problem; ND, not determined.

against T-47D, another breast cancer cell line, exhibited remarkably weak activity at all the three concentrations. Nevertheless, **JP-2** displayed potent inhibitory activity at 25 μM and **JP-10** and **-15** showed inhibitory potential to a very small extent. This loss of activity in case of T-47D cell line as compared to MCF-7 cell line can be attributed to the highly metastatic and aggressive nature of T-47D cell line in addition to the fact that it possesses proteins such as G1/S-specific cyclin-D3 and prohibitin, which stimulate cell growth, antiapoptosis, and carcinogenesis [29].

Structure–activity relationships

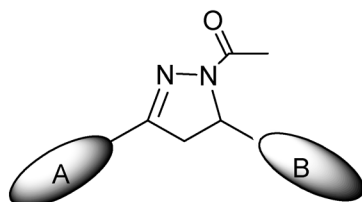
Structural–activity relationships emerged were based on the role played by ring A and ring B and substituents on them. Significant inhibitory potential was observed against the MCF-7 cells with phenyl on ring A (**JP-1–11**), which was also comparable to compounds bearing thiophene ring (**JP-14** and **-15**). The compounds with pyridyl as ring A were observed to be completely devoid of activity in both MCF-7 as T-47D cells (**JP-12**, **-13**, **-16**, and **-17**). Among the active compounds, comparing ring B of **JP-1**, **-2**, **-3**, **-4**, **-5** with **JP-11**, **-9**, **-10**, **-7**, and **-8**, respectively, it can be concluded that the thiophene

bearing compounds exhibited excellent inhibitory potential against the MCF-7 cells as compared to furan containing compounds. However, analogs containing phenyl ring (**JP-14** and **-15**) exhibited better activity than furan containing compounds. Among the thiophene containing derivatives the activity was better for bromo substitution (**JP-1**) than for chloro (**JP-4** and **-5**). Furan containing derivative with chloro substitution (**JP-7**) showed appreciable activity although only at higher concentration of 50 μM. Among the derivatives bearing electron releasing group, –CH₃ bearing thiophene derivative (**JP-2**) showed almost twice the activity seen with –OCH₃ (**JP-3**), but in case of furan containing compounds, –OCH₃ (**JP-10**) exhibited potent activity whereas –CH₃ (**JP-9**) exhibited no inhibitory potential. On the contrary, the MTT results obtained for the T-47D cell line suggested that the compounds bearing electron releasing group especially in –CH₃ bearing furan derivative (**JP-9**) exhibited better inhibitory potential than the electron withdrawing group (**JP-7** and **-11**). **JP-12**, a pyridine analogue, also showed comparable activity. These observations helped us to formulate a basic pharmacophore (Fig. 4) as shown below with the structural features required for potent anticancer activity.

Compounds are scavengers of intracellular reactive oxygen species (ROS)

Reactive oxygen species (ROS) and reactive nitrogen species are classified under unstable moieties, produced *in vivo* in low-to-moderate concentrations in response to normal cell metabolism [30–32]. Oxidative stress results as a consequence of the imbalance created when these substances are produced in higher concentrations [33]. Oxidative stress following a cascade of reactions eventually culminates in events having dire consequences such as damage to pyrimidine and purine bases, which may initiate the process of carcinogenesis [31].

In order to evaluate the antioxidant potential of the synthesized compounds, the representative derivatives **JP-1**, **-5**, **-8**, **-12**, and **-14** were evaluated using the H₂DCFDA dye, which gave an estimation of the intracellular ROS concentration (Fig. 5). In this assay, free radicals formation was first induced in primary human cells and then their scavenging by



MCF-7: Phenyl > Thiophene > Pyridyl
T-47D : Thiophene > Phenyl > Pyridyl

MCF-7: Thiophene > Phenyl > Furan
T-47D : Phenyl > Furan > Thiophene

Figure 4. Features decisive for the structure–activity relationships of the synthesized compounds.

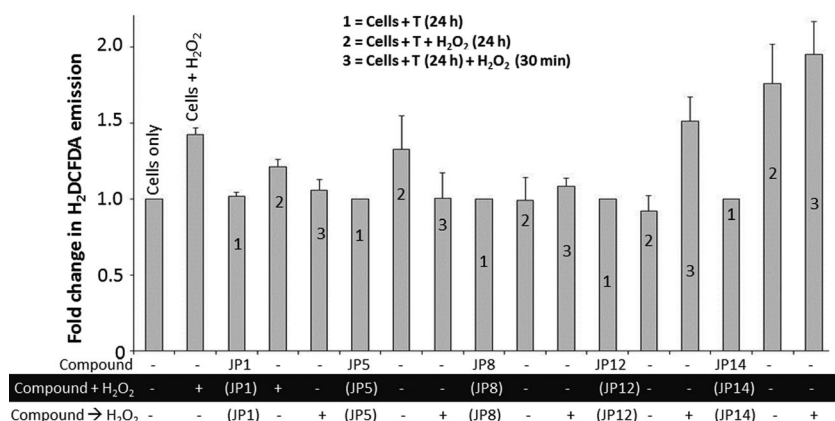


Figure 5. Graphical representation of the absorbance of the blood cells in response to the compounds **JP-5**, **-8**, **-12**, and **-14**. Antioxidant action of the compounds in case of pre- as well as post-treatment with either the compounds or H₂O₂.

the compounds was evaluated. H₂O₂ served as the free radical inducer while free radical production was studied using fluorescent H₂DCFDA stain by spectrophotometry. Among the tested compounds, **JP-5** was observed to exhibit significant antioxidant potential especially after 24 h treatment.

In Fig. 5, bar 1 represents the treatment of blood cells with the representative compounds only (**JP-1**, **-5**, **-8**, **-12**, and **-14**) whereas bar 2 represents the treatment of cells with the representative compounds followed by treatment with hydrogen peroxide for 24 h and bar 3 represents the incubation of cells with the representative compounds for 24 h followed by treatment with hydrogen peroxide after 30 min. In case of **JP-5**, addition of H₂O₂ to the cells immediately after treatment with the compounds resulted in generation of free radicals as indicated by the bar 2 but when the pre-treated cells were allowed to incubate for 24 h and then followed by the addition of H₂O₂, there was observed a marked decrease in the intracellular free radical concentration as depicted in bar 3. On the contrary, in case of **JP-8**, **-12**, and **-14**, even after incubating the cells with compound for 24 h, there was no decrease in the free radical concentration.

Compounds cause mitochondria-dependent cell death

Literature has evidences that cellular stresses, such as DNA damage and oxidative stress, trigger the release of caspase activating factors especially cytochrome-c [34]. The cytochrome-c released from the mitochondria result in the inception of a cascade of biochemical reactions culminating in cell death [35]. Numerous surveys have suggested that this release from the mitochondria and the mitochondrial depolarization occur simultaneously, thus making mitochondrial membrane potential a crucial factor not only for mitochondrial function but also for onset of apoptosis [36]. The membrane potential may range from high to low in healthy and apoptotic cells, respectively, thereby indicating mitochondria-dependent or -independent

cell death. Although various techniques are available for estimating the membrane potential, most commonly used method is employing cationic dyes such as **JC-1** (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide). These dyes redistribute between various compartments depending on the membrane potential forming multimeric units called J-aggregates. These J-aggregates are associated with the changes in the mitochondrial membrane potential. The dual fluorescence characteristics of the **JC-1** can be used in the assessment by comparing the relative fluorescence units (RFU) ratios of 590–600 nm (red)/527 nm (green). In the healthy cells, characterized by high potential, **JC-1** forms complexes exhibiting red fluorescence as opposed to the green in apoptotic cells [37]. When mitochondrial depolarization occurs, the red fluorescence decreases and the green fluorescence remains constant or increases, indicating cell death.

Two representative compounds with good IC₅₀ values, **JP-1** and **-14**, were selected in order to determine whether the cell death was mitochondria dependent or independent. For this purpose, the MCF-7 cell line was used and approximately 8000 cells were seeded and treated with 5 μM of the compounds. Consequently, **JC-1** dye was added to each of the wells and fluorescence read at 590 nm. A green fluorescence was observed, which confirmed that the treatments administered resulted in decreased mitochondrial membrane potential. At lower mitochondrial potential as in these cells, the **JC-1** dye fails to form the multimeric form, remaining in the monomeric form, thus resulting in green color. However, in the control cells, in the absence of synthesized compounds, the cells maintained their integrity without altering the mitochondrial membrane potential thus displaying red fluorescence. Figure 6 depicts that the RFU is significantly lower than that of control cells indicating that the compounds showed mitochondria-dependent cell death of the MCF-7 cells.

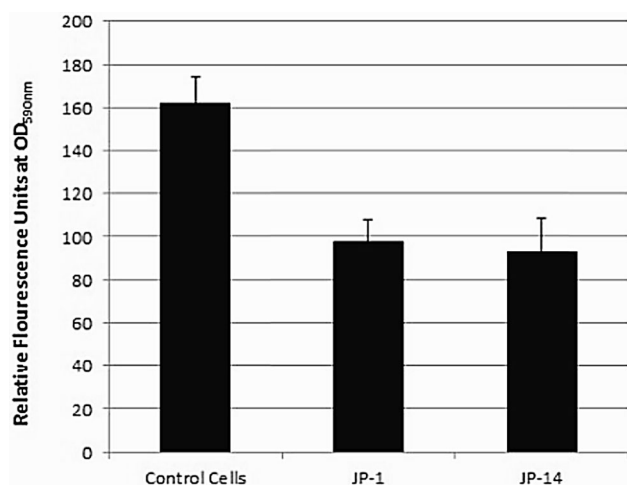


Figure 6. Graphical representation of the RFU of **JC-1** in control cells as compared to **JP-1** and **-14**. Data is expressed as mean values $\pm$ SD of three independent experiments.

Conclusions

We have designed and synthesized some 1-acetyl-4,5-dihydro-(1H)pyrazoles that have shown encouraging antiproliferative activity *in vitro* with low micromolar IC₅₀ values in MCF-7 breast cancer cell line. The compounds were found to be less cytotoxic to the normal cells. Our preliminary mechanistic interventions of biological activity suggested that these compounds possess the ability to scavenge the intracellular ROS, which are usually up-regulated in the carcinogenesis. Experimental evidences suggested that the investigated compounds were able to disrupt the potential of mitochondrial membrane, as evidenced by **JC-1** staining, resulting in mitochondrial depolarization indicating pro-apoptotic mechanism of breast cancer cell death. Further, detailed investigations of structure–activity relationships as well as explorations of antitumor activity are currently in progress. Compound **JP-1** has been identified for the further studies.

Experimental

Chemistry

General

All the reagents used in the synthetic process were of analytical grade, purchased from Sigma–Aldrich, Loba-Chemie Pvt. Ltd., S.D. Fine Chemicals, Sisco Research Laboratory. The completion of the reaction was ascertained by thin layer chromatography and the prepared plates were visualized by MAC Ultraviolet Fluorescence Analysis Cabinet. Melting points were recorded on Stuart SMP-30 melting point apparatus with open glass capillary tube and are uncorrected. Infrared spectra were recorded on Bruker IR spectrophotometer. Nuclear magnetic resonance (¹H and ¹³C) measurements were recorded on Bruker Advance II instrument at

400 and 100 MHz frequencies, respectively, using TMS ($\delta = 0$) as internal standard and HRMS experiment at SAIF, Panjab University, Chandigarh.

General procedure for the preparation of chalcones **JA-1–17**

To a stirred mixture of an aryl or heteroaryl ketone (**1**; 1 equiv.) and an aryl or heteroaryl aldehyde (**2**; 1 equiv.) in methanol, sodium hydroxide (5%, 4 mL) was added at 0°C. The mixture was stirred at room temperature for about 15 min to 24 h (TLC). Solid was obtained after filtration and was recrystallized from methanol to afford the pure chalcone [26].

General procedure for the preparation of 1-acetyl-3,5-diaryl-4,5-dihydro(1H)pyrazoles (**JP-1–17**)

To a solution of an appropriate chalcone (**JA-1–17**; 1 mmol, 1 equiv.) in 5 mL acetic acid, hydrazine hydrate 80% (1.5 mmol) was added. The mixture was refluxed for about 4–6 h (TLC). Then the mixture was poured into ice water mixture to get crude pyrazole derivatives, which were then purified by recrystallization from methanol to afford the pure compounds (**JP-1–17**).

1-(3-(4-Bromophenyl)-5-(thiophen-2-yl)-4,5-dihydro(1H)-pyrazol-1-yl)ethanone (**JP-1**) [38]

Light yellow solid; yield: 65%; m.p.: 164–165°C; λ_{max} (MeOH): 325 nm. IR (KBr cm⁻¹): 3014 (C–H), 1660 (C=O), 1591 (C=N stretch), 1246 (C–N stretch), 1136 (C–S stretch). ¹H NMR (400 MHz, CDCl₃, TMS = 0) δ : 7.61 (2H, d, $J = 8.64$ Hz), 7.57 (2H, d, $J = 8.62$ Hz), 7.18 (1H, dd, $J = 1.04$ and 5.08 Hz), 7.01 (1H, d, $J = 3.36$ Hz), 6.92 (1H, dd, $J = 3.56$ and 5.08 Hz), 5.91 (1H, dd, $J = 4.04$ and 11.48 Hz), 3.70 (1H, dd, $J = 11.48$ and 17.6 Hz), 3.31 (1H, dd, $J = 4.08$ and 17.6 Hz), 2.38 (3H, s).

1-(5-(Thiophen-2-yl)-3-(p-tolyl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (**JP-2**) [39]

Yellow solid; yield: 55%; m.p.: 180–182°C; λ_{max} (MeOH): 304 nm. IR (KBr cm⁻¹): 3007 (C–H stretch), 1660 (C=O), 1609 (C=N stretch), 1587 (C=C aromatic), 1176 (C–N stretch), 1014 (C–S stretch). ¹H NMR (400 MHz, CDCl₃, TMS = 0) δ : 7.64 (2H, d, $J = 8.32$ Hz), 7.25 (2H, d, $J = 8.32$ Hz), 7.17 (1H, dd, $J = 1.0$ and 5.04 Hz), 7.01 (1H, d, $J = 3.36$ Hz), 6.91 (1H, m), 5.89 (1H, dd, $J = 3.9$ and 11.4 Hz), 3.70 (1H, dd, $J = 11.4$ and 17.56 Hz), 3.33 (1H, dd, $J = 3.9$ and 17.56 Hz), 2.40 (3H, s), 2.39 (3H, s).

1-(3-(4-Chlorophenyl)-5-(thiophen-2-yl)-4,5-dihydro(1H)-pyrazol-1-yl)ethanone (**JP-4**) [38]

Yellow solid; yield: 67%; m.p.: 125–127°C; λ_{max} (MeOH): 307 nm. IR (KBr cm⁻¹): 3010 (C–H stretch), 1660 (C=O), 1593 (C=N stretch), 1215 (C–N stretch), 1142 (C–S), 702 (C–Cl stretch). ¹H NMR (400 MHz, CDCl₃, TMS = 0) δ : 7.69 (2H, d, $J = 8.42$ Hz), 7.41 (2H, d, $J = 8.42$ Hz), 7.39 (1H, s), 7.18 (1H, dd, $J = 1.0$ and 5.08 Hz), 7.01 (1H, d, $J = 3.36$ Hz), 6.94 (1H, m), 5.92 (1H, dd, $J = 4.0$ and 11.44 Hz), 3.70 (1H, dd, $J = 11.48$ and 17.6 Hz), 3.31 (1H, dd, $J = 4.0$ and 17.56 Hz), 2.39 (3H, s).

1-(3-Phenyl-5-(thiophen-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (**JP-6**) [39]

Dark brown solid; yield: 61%; m.p.: 110–112°C; λ_{max} (MeOH): 249 nm. IR (KBr cm⁻¹): 2925 (C–H stretch), 1658 (C=O), 1620 (C=N

stretch), 1523 (C=C aromatic), 1232 (C–N stretch), 1090 (C–S stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.70 (2H, d, $J = 2.32$ Hz), 7.46 (3H, m), 7.19 (1H, dd, $J = 1.12$ and 5.08 Hz), 7.03 (1H, d, $J = 3.52$ Hz), 6.93 (1H, dd, $J = 3.52$ and 5.04 Hz), 5.9 (1H, dd, $J = 3.76$ and 11.44 Hz), 3.9 (1H, dd, $J = 11.48$ and 18.08 Hz), 3.5 (1H, dd, $J = 3.8$ and 18.04 Hz), 2.3 (3H, s).

1-(5-(Furan-2-yl)-3-(*p*-tolyl)-4,5-dihydro(1H)pyrazol-1-yl)-ethanone (JP-9) [39]

Light brown solid; yield: 70%; m.p.: 120–121°C; λ_{max} (MeOH): 297 nm. IR (KBr cm^{-1}): 2923 (C–H stretch), 1661 (C=O), 1640 (C=N stretch), 1591 (C=C aromatic), 1250 (C–O stretch), 1210 (C–N stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.66 (1H, s), 7.63 (1H, s), 7.29 (1H, s), 7.24 (1H, s), 7.25 (1H, s), 6.31 (1H, s), 6.30 (1H, d, $J = 1.4$ Hz), 5.68 (1H, dd, $J = 4.72$ and 11.64 Hz), 3.56 (1H, dd, $J = 11.64$ and 17.48 Hz), 3.42 (1H, dd, $J = 4.76$ and 17.48 Hz), 2.40 (3H, s), 2.38 (3H, s).

1-(3-(4-Bromophenyl)-5-(furan-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (JP-11) [40]

Light brown solid; yield: 68%; m.p.: 141–143°C; λ_{max} (MeOH): 393 nm. IR (KBr cm^{-1}): 3011 (C–H stretch), 1659 (C=O), 1594 (C=N stretch), 1532 (C=C aromatic), 1252 (C–O stretch), 1215 (C–N stretch), 754 (C–Br stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.62 (1H, s), 7.61 (1H, s), 7.56 (1H, s), 7.55 (1H, s), 7.29 (1H, m), 6.32 (1H, s), 6.31 (1H, s), 5.69 (1H, dd, $J = 4.84$ and 11.76 Hz), 3.56 (1H, dd, $J = 11.72$ and 17.44 Hz), 3.41 (1H, dd, $J = 4.88$ and 17.48 Hz), 2.38 (3H, s).

1-(5-(4-Chlorophenyl)-3-(thiophen-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (JP-14) [41]

Yellow solid; yield: 77%; m.p.: 107–109°C; λ_{max} (MeOH): 317 nm. IR (KBr cm^{-1}): 3007 (C–H stretch), 1657 (C=O), 1632 (C=N stretch), 1523 (C=C aromatic), 1232 (C–N stretch), 1089 (C–S stretch), 709 (C–Cl stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.44 (1H, dd, $J = 1.08$ and 5.08 Hz), 7.28 (1H, s), 7.21 (1H, dd, $J = 1.04$ and 3.68 Hz), 7.18 (1H, s), 7.16 (1H, s), 7.16 (1H, s), 7.07 (1H, dd, $J = 3.68$ and 5.0), 5.55 (1H, dd, $J = 4.68$ and 11.84 Hz), 3.76 (1H, dd, $J = 11.84$ and 17.48 Hz), 3.18 (1H, dd, $J = 4.72$ and 17.52 Hz), 2.38 (3H, s).

1-(5-Phenyl-3-(thiophen-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)-ethanone (JP-15) [42]

Yellow solid; yield: 56%; m.p.: 118–120°C; λ_{max} (MeOH): 311 nm. IR (KBr cm^{-1}): 3013 (C–H stretch), 1655 (C=O), 1590 (C=N stretch), 1523 (C=C aromatic), 1141 (C–S stretch), 1215 (C–N stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.43 (1H, dd, $J = 1.04$ and 5.04 Hz), 7.32 (1H, d, $J = 1.16$), 7.30 (1H, d, $J = 1.28$ Hz), 7.26 (1H, s), 7.24–7.20 (3H, m), 7.06 (1H, dd, $J = 3.68$ and 5.08 Hz), 5.59 (1H, dd, $J = 4.56$ and 11.76 Hz), 3.76 (1H, dd, $J = 11.84$ and 17.48 Hz), 3.15 (1H, dd, $J = 4.60$ and 11.44 Hz), 2.39 (3H, s).

1-(5-Phenyl-3-(pyridin-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)-ethanone (JP-16) [43]

Yellow solid; yield: 60%; m.p.: 135–137°C; λ_{max} (MeOH): 304 nm. IR (KBr cm^{-1}): 3007 (C–H stretch), 1664 (C=O), 1581 (C=N stretch), 1561 (C=C aromatic), 1253 (C–N stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 8.61 (1H, m), 8.10 (1H, d, $J = 7.92$), 7.78–7.4 (1H, m), 7.33 (1H, d, $J = 2.16$ Hz), 7.31 (1H, d, $J = 1.56$ Hz), 7.29 (1H, s), 7.25 (1H, s), 7.23 (1H, d, $J = 6.28$ Hz), 7.22 (1H, s), 5.61 (1H, dd,

$J = 4.76$ and 12 Hz), 3.86 (1H, dd, $J = 11.96$ and 18.56 Hz), 3.38 (1H, dd, $J = 4.76$ and 18.6 Hz), 2.44 (3H, s).

1-(3-(Pyridin-2-yl)-5-(thiophen-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (JP-17) [44]

Yellow solid; yield: 55%; m.p.: 113–114°C (120–121°C); λ_{max} (MeOH): 304 nm. IR (KBr cm^{-1}): 3013 (C–H stretch), 1657 (C=O), 1585 (C=N stretch), 1522 (C=C aromatic), 1148 (C–S stretch), 1215 (C–N stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 8.63–8.61 (1H, m), 8.09 (1H, d, $J = 8.0$ Hz), 7.70 (1H, d, $J = 7.72$ Hz), 7.34–7.30 (1H, m), 7.17 (1H, d, $J = 6.2$ Hz), 7.03 (1H, s), 6.91 (1H, d, $J = 5.04$ Hz), 5.92 (1H, dd, $J = 4.20$ and 11.52 Hz), 3.82 (1H, dd, $J = 11.56$ and 18.52 Hz), 3.59 (1H, dd, $J = 4.20$ and 18.52 Hz), 2.41 (3H, s). ^{13}C NMR (100 MHz, CDCl_3 , TMS = 0) δ : 169.1, 155.4, 150.6, 149.4, 144.1, 136.3, 126.7, 124.8, 124.6, 124.4, 121.2, 55.5, 41.7, 22.0.

The following compounds were found to be unreported.

1-(3-(4-Methoxyphenyl)-5-(thiophen-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (JP-3)

Light brown; yield: 56%; m.p.: 89–90°C; λ_{max} (MeOH): 315 nm. IR (KBr cm^{-1}): 3007 (C–H stretch), 1657 (C=O), 1608 (C=N stretch), 1517 (C=C aromatic), 1113 (C–S stretch), 1253 (C–O stretch), 1216 (C–N stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.7 (1H, d, $J = 9.64$ Hz), 7.69 (1H, d, $J = 2.0$ Hz), 7.22 (1H, d, $J = 5.08$ Hz), 7.16 (1H, dd, $J = 1.2$ Hz), 6.90–6.94 (2H, m), 6.95 (1H, s), 5.89 (1H, dd, $J = 3.88$ and 11.36 Hz), 3.69 (1H, dd, $J = 11.36$ and 17.44 Hz), 3.31 (1H, dd, $J = 3.96$ and 17.48 Hz), 3.85 (3H, s), 2.38 (3H, s). HRMS (TOF-ESI) calcd. for $\text{C}_{16}\text{H}_{16}\text{N}_2\text{O}_2\text{S}$, 300.0932 (M^+); observed: 301.1133 ($\text{M}+\text{H}^+$).

1-(3-(2,4-Dichlorophenyl)-5-(thiophen-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (JP-5)

Yellow solid; yield: 85%; m.p.: 115–116°C; λ_{max} (MeOH): 293 nm. IR (KBr cm^{-1}): 3012 (C–H stretch), 1658 (C=O), 1609 (C=N stretch), 1589 (C=C aromatic), 1176 (C–S stretch), 1215 (C–N), 702 (C–Cl stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.7 (1H, d, $J = 8.48$ Hz), 7.46 (1H, s), 7.3 (1H, s), 7.19 (1H, dd, $J = 1.08$ and 5.04 Hz), 7.03 (1H, d, $J = 3.2$ Hz), 6.93 (1H, m), 5.91 (1H, dd, $J = 3.76$ and 11.44 Hz), 3.91 (1H, dd, $J = 11.48$ and 18.08 Hz), 3.49 (1H, dd, $J = 3.80$ and 18.04 Hz), 2.37 (3H, s). HRMS (TOF-ESI) calcd. for $\text{C}_{15}\text{H}_{12}\text{Cl}_2\text{N}_2\text{OS}$, 338.0047 (M^+); observed: 339.1031 ($\text{M}+\text{H}^+$).

1-(3-(4-Chlorophenyl)-5-(furan-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (JP-7)

Whitish yellow solid; yield: 72%; m.p.: 156–157°C; λ_{max} (MeOH): 297 nm. IR (KBr cm^{-1}): 3010 (C–H stretch), 1663 (C=O), 1596 (C=N stretch), 1502 (C=C aromatic), 1252 (C–O stretch), 1215 (C–N stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.70 (1H, d, $J = 2.36$ Hz), 7.68 (1H, d, $J = 1.96$ Hz), 7.41 (1H, s), 7.39 (1H, d, $J = 2.4$ Hz), 7.30 (1H, m), 6.33 (1H, s), 6.31 (1H, t), 5.69 (1H, dd, $J = 4.84$ and 11.72 Hz), 3.56 (1H, dd, $J = 11.72$ and 17.52 Hz), 3.41 (1H, dd, $J = 4.84$ and 17.52 Hz), 2.32 (3H, s). HRMS (TOF-ESI) calcd. for $\text{C}_{15}\text{H}_{13}\text{ClN}_2\text{O}_2$, 288.0666 (M^+); observed: 289.0046 ($\text{M}+\text{H}^+$).

1-(3-(2,4-Dichlorophenyl)-5-(furan-2-yl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (JP-8)

Yellow solid; yield: 64%; m.p.: 115–116°C; λ_{max} (MeOH): 295 nm. IR (KBr cm^{-1}): 3013 (C–H stretch), 1711 (C=O), 1665 (C=N aromatic), 1586 (C=C aromatic), 1257 (C–O stretch), 1215 (C–N

stretch), 751 (C–Cl stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.75 (1H, d, J = 8.48 Hz), 7.46 (1H, s), 7.32–7.29 (2H, m), 6.33 (1H, s), 6.32 (1H, m), 5.67 (1H, dd, J = 4.56 and 11.8 Hz), 3.79 (1H, dd, J = 11.8 and 17.96 Hz), 3.57 (1H, dd, J = 4.96 and 17.92 Hz), 2.36 (3H, s). HRMS (TOF-ESI) calcd. for $\text{C}_{15}\text{H}_{12}\text{Cl}_2\text{N}_2\text{O}_2$, 322.0276 (M) $^+$; observed: 345.990 ($\text{M}+\text{Na}$) $^+$.

1-(5-(Furan-2-yl)-3-(4-methoxyphenyl)-4,5-dihydro(1H)-pyrazol-1-yl)ethanone (JP-10)

Dark brown solid; yield: 59%; m.p.: 122–124°C; λ_{max} (MeOH): 301 nm. IR (KBr cm^{-1}): 3005 (C–H stretch), 1659 (C=O), 1606 (C=N stretch), 1596 (C=C aromatic), 1250 (C–O stretch), 1223 (C–N stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 7.71 (1H, d, J = 2.0 Hz), 7.69 (1H, d, J = 2.04 Hz), 7.29 (1H, m), 6.95 (1H, d, J = 2.0 Hz), 6.93 (1H, d, J = 2.0 Hz), 6.31 (1H, d, J = 1.48 Hz), 6.30 (1H, d, J = 1.44 Hz), 5.67 (1H, dd, J = 4.68 and 11.6 Hz), 3.85 (3H, s), 3.55 (1H, dd, J = 11.56 and 17.36 Hz), 3.41 (1H, dd, J = 4.68 and 17.36 Hz), 2.38 (3H, s). HRMS (TOF-ESI) calcd. for $\text{C}_{16}\text{H}_{16}\text{N}_2\text{O}_3$, 284.1161 (M) $^+$; observed: 285.1680 ($\text{M}+\text{H}$) $^+$.

1-(5-(2,5-Dimethoxy)-3-phenyl-4,5-dihydro(1H)pyrazol-1-yl)ethanone (JP-12)

Yellow solid; yield: 74%; m.p.: 112–114°C; λ_{max} (MeOH): 328 nm. IR (KBr cm^{-1}): 3015 (C–H stretch), 1660 (C=O), 1583 (C=N stretch), 1215 (C–N stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 8.86 (1H, d, J = 1.68 Hz), 8.64 (1H, dd, J = 1.56 and 4.80 Hz), 8.09–8.06 (m, 1H), 7.36–7.33 (1H, m), 6.82 (1H, d, J = 8 Hz), 6.75–6.73 (m, 1H), 6.58 (1H, d, J = 2 Hz), 5.83 (1H, dd, J = 4.68 and 11.84 Hz), 3.04 (1H, dd, J = 4.72 and 17.72 Hz), 3.41 (1H, dd, J = 11.92 and 17.32 Hz), 3.81 (3H, s), 3.72 (3H, s), 2.46 (3H, s). HRMS (TOF-ESI) calcd. for $\text{C}_{18}\text{H}_{19}\text{N}_3\text{O}_3$, 325.1430 (M) $^+$; observed: 348.0940 ($\text{M}+\text{H}$) $^+$.

1-(5-(Furan-2-yl)-3-(pyridin-3-yl)-4,5-dihydro(1H)pyrazol-1-yl)ethanone (JP-13)

Yellow solid; yield: 63%; m.p.: 95–97°C; λ_{max} (MeOH): 301 nm. IR (KBr cm^{-1}): 3011 (C–H stretch), 1662 (C=O), 1595 (C=N stretch), 1553 (C=C aromatic), 1256 (C–O stretch), 1215 (C–N stretch). ^1H NMR (400 MHz, CDCl_3 , TMS = 0) δ : 8.92 (1H, d, J = 1.64), 8.67 (1H, dd, J = 1.48 and 4.76 Hz), 8.10 (1H, t, J = 1.84 Hz), 7.40–7.36 (1H, m), 7.30 (1H, m), 6.35 (1H, m), 6.32 (1H, m), 5.72 (1H, dd, J = 4.92 and 11.76 Hz), 3.61 (1H, dd, J = 11.76 and 17.56 Hz), 3.47 (1H, dd, J = 4.88 and 11.76 Hz), 2.39 (3H, s). HRMS (TOF-ESI) calcd. for $\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_2$, 255.1008 (M) $^+$; observed: 256.0901 ($\text{M}+\text{H}$) $^+$.

Biology

Cell culture and treatment

All the cell lines were procured from National Cell Repository, NCCS, Pune. A-549 and H-460 (liver) and MCF-7 and T-47D (breast) cell lines representing different human cancers were grown in DMEM media supplemented with 10% fetal bovine serum (FBS) and antibiotic solution (1% Penstrip, all the reagents from Invitrogen). Cells were cultured in DMEM media with 10% FBS, 50 U/mL penicillin G and 50 $\mu\text{g}/\text{mL}$ streptomycin sulfate. The cells were incubated at 37°C with 5% CO_2 and 95% humidity conditions. For experiments, cells were seeded in equal numbers after Trypan blue cell counting (8000 cells per well of 96-well plate). The compounds were dissolved in cell culture grade DMSO up to concentration of 100 mM and further dilutions were done in serum free DMEM media. The total amount of media per well

(200 μL per well of 96-well plate) was kept constant and all the treatment volumes were accommodated within these ranges only.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MTT assay was carried out in 96-well plates where total volume of media was 200 $\mu\text{L}/\text{well}$ [27]. Briefly cells after the treatments were washed with 1% PBS and were mixed with 100 $\mu\text{L}/\text{mL}$ well of MTT (5 mg in 10 mL of 1% PBS) and incubated at room temperature in dark for 4 h to allow formation of formazan crystals. Each well was then mixed with 100 μL of DMSO to dissolve the crystals followed by ELISA readings at 570 nm. The results were then represented as mean $\pm$ SD obtained from three independent experiments.

Antioxidant assay

Five milliliters of human blood was taken and processed with RBC lysis buffer to get the lymphocytes and counting was done using Trypan blue assay in the automated cell counter, 10,000 cells were seeded into 12 wells of the microtiter plate [27]. The compounds to be evaluated were added in triplicate to the respective cells in concentration such that the final concentration of each well would be 5 μM . Briefly cells were incubated for 24 h followed by treatment either with compounds alone, compounds + H_2O_2 or H_2O_2 alone for 24 h followed by other treatments. After the treatments, 4 μL of H_2DCFDA dye was added in the cultured cells and kept in dark for 30 min followed by absorbance at emission wavelength of 530 nm. All the treatments were done in triplicates.

Determination of mitochondrial membrane potential

Experimental and control cell populations were counted to be 5×10^5 cells/mL using the Trypan blue counting method, from which a small aliquot was taken and the cell density measured. The cells were concentrated to less than 3×10^6 cells/mL. In order to obtain this target concentration, the cells were centrifuged at <300g for 5–8 min. After discarding the supernatant enough culture media was added and the cells were resuspended by gentle vortexing. One milliliter per sample is placed into 15 mL polypropylene centrifuge tubes and the sample are stained with 0.5 mL $1 \times$ working solution of MitoPT $^{\text{®}}$ after centrifuging and gentle mixing. This is then incubated 15–20 min at 37°C in the dark. Cells were washed to remove excess MitoPT. Then the cells are centrifuged and pelleted. Discarding the supernatant 2 mL $1 \times$ assay buffer or culture media is added. For the second resuspension, the cells are centrifuged and pelleted. Discarding the supernatant 0.5–1 mL $1 \times$ assay buffer or culture media is added. Pipetted out 100–200 μL of each sample per well (in triplicate) into a black round or flat-bottom 96-well microtiter plate.

Jurkat and HL-60 cells were exposed to DMSO as the negative control (left, dark orange bars) or 50 μM CCCP depolarizing agent (right, light orange bars) for 15 min at 37°C, subsequently incubated with MitoPT $^{\text{®}}$ JC-1 for 20 min at 37°C, and washed. 100 μL aliquots were analyzed in triplicate in a black 96-well plate using a Molecular Devices Gemini XS fluorescence plate reader set at 488 nm excitation and 590 nm emission filter settings. The amount of orange fluorescence was measured by the plate reader. Healthy cells in the DMSO control populations exhibited a high level of orange fluorescence; metabolically stressed cells in the

CCCP-stimulated samples exhibited a reduced level of orange fluorescence after the mitochondria became depolarized. As the membrane potential gradient collapses, JC-1 will equilibrate out of the mitochondria and into the cytosol, causing cells to lose their red fluorescence.

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