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Imidazole derivatives show anticancer potential by inducing apoptosis and cellular senescence†

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Imidazole-based compounds are attractive targets in the design of novel chemical structures for the discovery of new drugs. In the current study, we have synthesized a series of new 2,4,5-trisubstituted and 1,2,4,5-tetrasubstituted imidazoles by multicomponent reaction (MCR). Vanillin and isovanillin derivatives were reacted with benzil/pyridil and diverse amines and ammonium acetate in acetic acid at 50–110 °C for 24 h to afford respective imidazoles in 55–70% yields. The series of molecules were evaluated for anti-cancer potential against the National Cancer Institute's 60 human cancer cell line panel. Preliminary screening highlighted the anticancer potential of 2,2′-(2-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-isobutyl-1*H*-imidazole-4,5-diyl)dipyridine (**NSC 771432**) against different cancer cell types. A549 cells were treated *in vitro* to determine the mode of action of **NSC 771432** on growth of these cells. This compound inhibits anchorage independent growth and cell migration, and induces cell cycle arrest in the G2/M phase. Also, the exposure of A549 cells to **NSC 771432** leads to cellular senescence.

1. Introduction

Cancer is the most prevalent disease worldwide and one of the leading causes of increased mortality. Every year, about 12 million new cases of cancer are reported. Among all cancers, about 40% of diagnosed cases include lung, breast, colorectal and stomach cancers.[1] Of all cancers, approximately 47% of cancer cases and 55% of cancer deaths are registered in underdeveloped countries with a low or a medium Human Development Index (HDI). At the current global rate of incidence for cancers it is estimated that approximately 22 million new cancer patients will be registered per year by 2030.[2] These statistics clearly warrant the need for development of novel anticancer agents for effective treatment of different cancers.

In the recent past, applications of imidazole derivatives in medicinal chemistry have achieved remarkable progress. The structural features of the imidazole ring with desirable electron rich characteristics are beneficial for imidazole derivatives to readily bind with a variety of enzymes and receptors in the biological system. 2,4,5-Trisubstituted and 1,2,4,5-tetrasubstituted imidazoles[3] are present in compounds possessing versatile pharmacological activities such as p38 MAP kinase inhibitors,[4] antitumor agents,[5] B-Raf kinase inhibitors,[6] antibacterial agents,[7] cannabinoid receptor antagonists,[8] anti-inflammatory agents,[9] CSBP kinase inhibitors,[10] and glucagon receptor antagonists.[11] The imidazole derivatives readily bind with a variety of enzymes and protein molecules and receptors compared with the other heterocyclic rings. This potency and wide applicability of the imidazole pharmocophore can be attributed to its hydrogen bond donor–acceptor capability, π–π stacking interactions, co-ordination bonds with metals (*e.g.*, Mg, Fe, and Zn) as a ligands, van der Waals, polarization and hydrophobic forces. Imidazoles could interfere with DNA synthesis, and then halt cell growth and division. Several imidazole containing molecules have been reported to show cytotoxic activity against diverse cancer cell lines.[12] In the present study, we report the synthesis of imidazole containing compounds by multi-component reaction and their anti-cancer potential against the panel of NCI 60 cancer cell lines representing diverse histologies such as leukemia, melanoma, and cancers of lung, colon, kidney, ovary, breast, prostate, and central nervous system. The molecule showing good anti-proliferative properties against many different cancer cell types was evaluated by *in vitro* assays to determine the mode of its action.

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2. Results and discussion

2.1. Chemistry

Of the several methods reported in the literature for the synthesis of 2,4,5-trisubstituted imidazoles and 1,2,4,5-tetrasubstituted imidazoles, the three or four component one pot condensation of aryl glyoxals, aldehydes, ammonium acetate and amines in refluxing acetic acid was found to be convenient method[13] as shown in Scheme 1. Thus commercially available vanillin (**1a**) and isovanillin (**1b**) were treated with cycloalkyl methane sulfonates (cyclopentyl and cyclohexyl) and K_2CO_3 in toluene at reflux for 24 h to afford substituted benzaldehydes[14–17] **2a**, **2c**, **2b** and **2d**, respectively, in good yields (Scheme 1). The synthesis route for the new imidazole derivatives involves the treatment of aldehydes **2a–2d** independently with an alkyl amine, ammonium acetate, benzil or pyridil to yield **3–9**, **11**, **12** or **10**, the substituted imidazoles in good yields (Scheme 1) (Fig. 1).

2.2. Biological study

Anti-cancer activity of imidazoles. Details of the methodology for NCI 60 cell line screening were described earlier.[18] Briefly, the panel is organized into nine subpanels representing diverse histologies: leukemia, melanoma, and cancers of lung, colon, kidney, ovary, breast, prostate, and central nervous system. The cells are grown in supplemented RPM1 1640 medium for 24 h. The test compounds were dissolved in DMSO and incubated with cells at five concentrations with 10-fold dilutions, the highest being 10^{-4} M and the others being 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M. The assay is terminated by addition of cold trichloroacetic acid, and the cells are fixed and stained with sulforhodamine B. Bound stain is solubilised, and the absorbance is read on an automated plate reader. The cytostatic parameter 50% growth inhibition (GI_{50}) was calculated from time zero, control growth and the five concentration level absorbance. The cytotoxic parameter that is, inhibitory concentrations (LC_{50}) represent the average of two independent experiments. The *in vitro* screening is a two-stage process started with the evaluation of the compound against the 60 human tumor cell lines with a single dose of 10.0 μM, which is done by following the same protocol as for five dose screening. Only the compounds that show more than 60% of growth inhibition in at least 8 tumor cell lines are selected for further testing and the others were assumed inactive. As per the selection criteria by the NCI screening team, all new compounds are first compared with the existing database and a subset of novel compound(s) is screened first. The same criteria were applied for selection in the present study and 10 of our compounds were selected initially for a single dose screening at 10 μM. In this paper we present the results of compounds selected and studied by NCI in the first selection process. A comparative summary of the % growth inhibition by all compounds is shown as a heat map in Fig. 2.

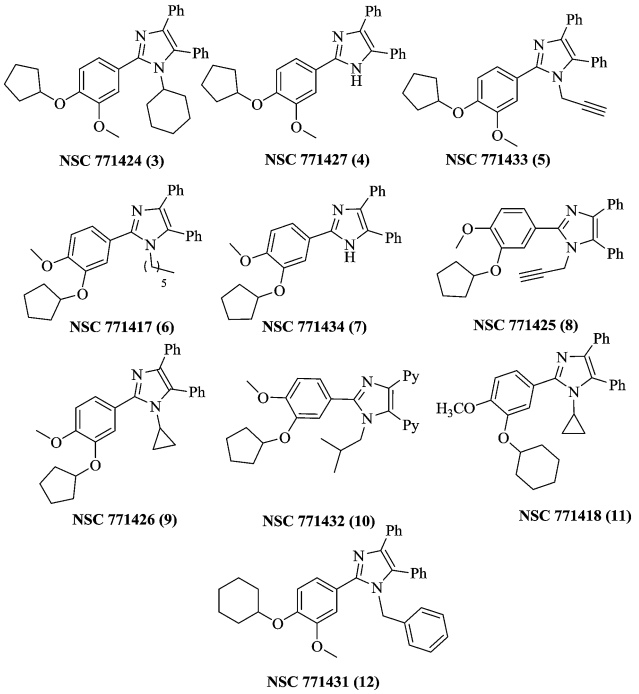
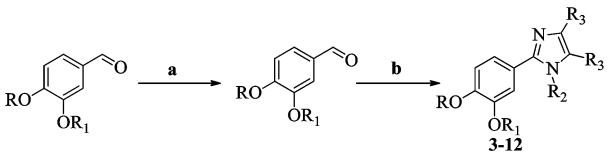


Fig. 1 Structures of imidazoles **3–12** and NCI annotations.



R = H, R_1 = CH_3 **1a**; R = CH_3, R_1 = H **1b**

R = cyclopentyl, R_1 = CH_3 **2a**; R = CH_3, R_1 = cyclopentyl **2b**; R = cyclohexyl, R_1 = CH_3 **2c**; R = CH_3, R_1 = cyclohexyl **2d**

R = cyclopentyl, R_1 = CH_3, R_3 = Ph

3 R_2= cyclohexyl; **4** R_2 = H; **5** R_2 = propargyl

R = CH_3, R_1 = cyclopentyl, R_3 = Ph

6 R_2= hexyl; **7** R_2 = H; **8** R_2 = propargyl; **9** R_2 = cyclopropyl

10 R = CH_3, R_2= isobutyl, R_1 = cyclopentyl, R_3 = Py

11 R = CH_3, R_1 = cyclohexyl, R_2 = cyclopropyl, R_3 = Ph

12 R = cyclohexyl, R_1 = CH_3, R_2 = benzyl, R_3 = Ph

Reagents and conditions: a) cycloalkyl methanesulfonate, K_2CO_3, toluene, reflux, 12h; b) benzil (or) pyridil, alkyl amine, ammonium acetate, AcOH, reflux, 24 h.

Scheme 1 Synthesis of imidazoles **3–12**.

As can be seen from Fig. 2, with the exception of **NSC 771432**, all other compounds had only low to moderate effects on the growth inhibition of most cell lines. **NSC 771432** was very effective in causing protein growth inhibition on majority of the cell lines. Therefore, it was evaluated at five concentration levels (100, 10, 1.0, 0.1 and 0.01 μM). The mean values of GI_{50} and LC_{50} on all 60 cell lines are given in Table S1 (ESI†).

A high LC_{50} value compared to the GI_{50} value for **NSC 771432** suggests a wide therapeutic window for its further development. This compound has a high inhibitory effect on selected cell lines (CCRF-CEM; K-562; A549; HCT-116; HCT-15; SF-295; NCI/

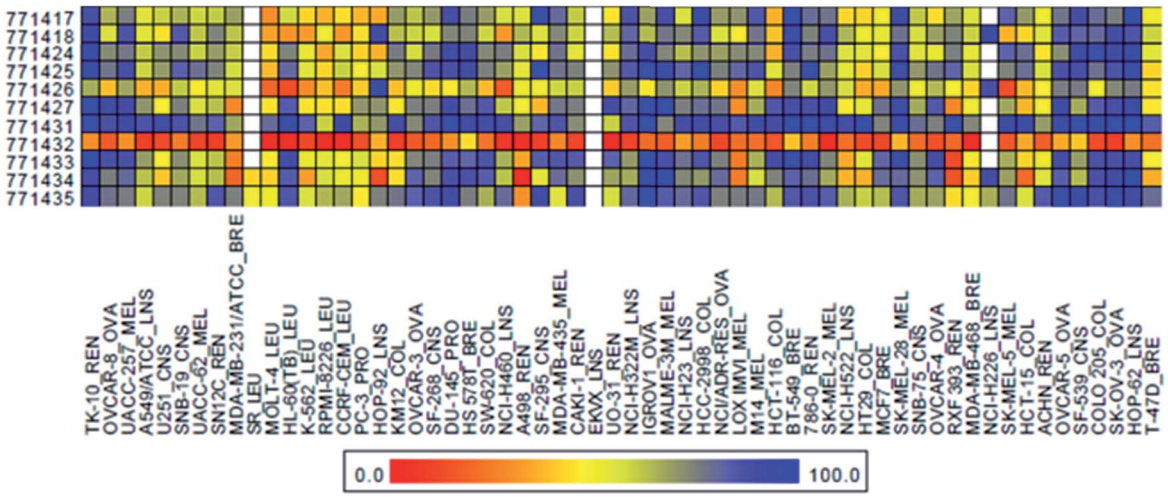


Fig. 2 Heat map displaying cytotoxic potential of series of imidazoles prepared. **NSC 771432** showed more cytotoxic potential against a large number of cell lines in the NCI60 cell line panel representing common tumors.

ADR-RES; ACHN; UO-31; MCF7; HS 578T and T-47D). The complete data of GI_{50} and LC_{50} values are given in the ESI.† Fig. 3 shows dose response curves of each cancer panel. To understand the cytotoxic potential and to gain more insights into **NSC 771432** activity, further studies were carried out on A549, a non-small lung cancer cell line.

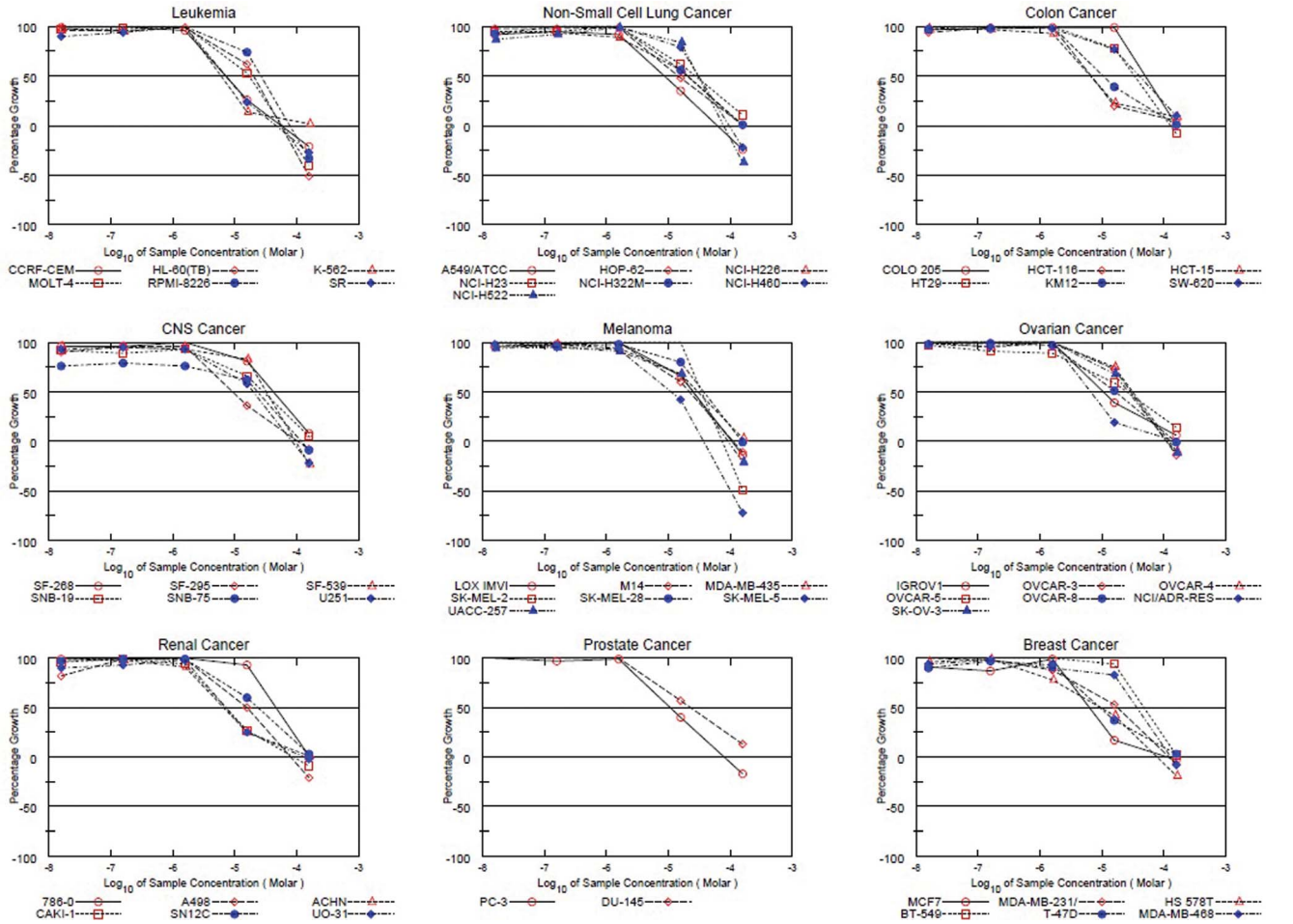


Fig. 3 Effect of **NSC 771432** on the viability of cancer cells of different cancer types. Selected cells were treated with 5 different concentrations (0.01 to 100 μM) of **NSC 771432** for 48 h and the cell survival was assessed with SRB assays ($n = 3$).

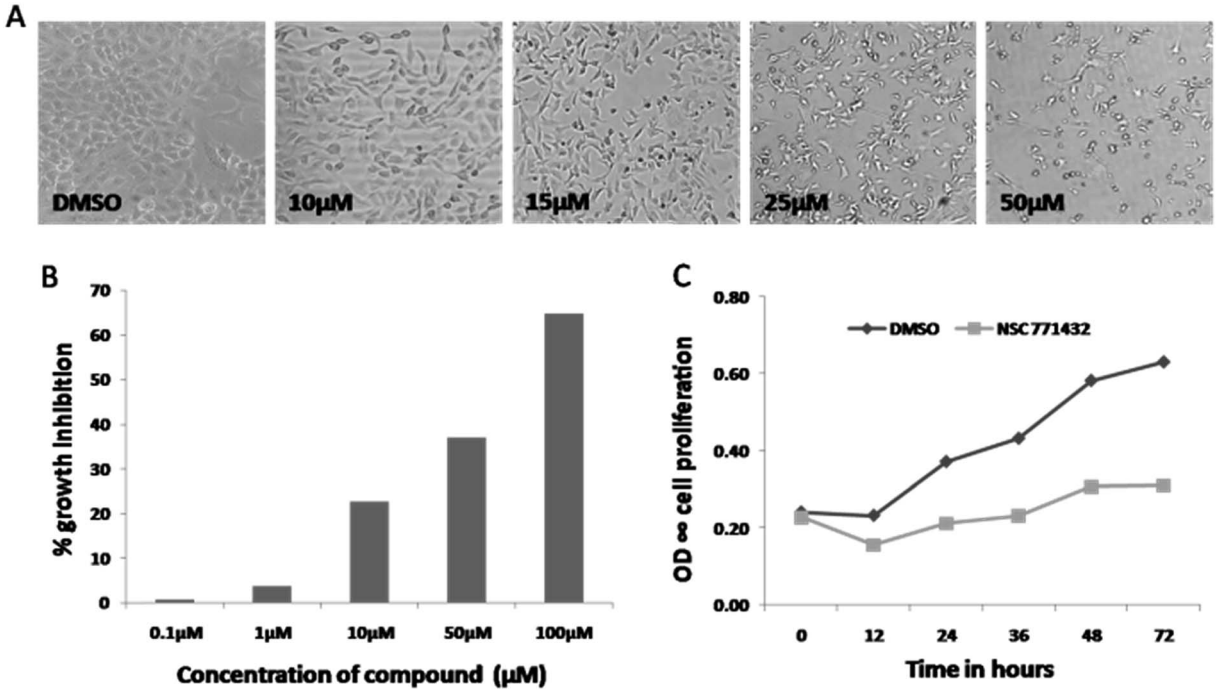


Fig. 4 (A) A549 cells were treated with compound **NSC 771432** at indicated concentration or DMSO. On exposure of A549 cells to NSC 771432 the extent of change in the morphology of cells observed with increasing concentration. (B) Concentration dependent inhibition of A549 cell proliferation by **NSC 771432**. (C) Time course to observe the effect of **NSC 771432** on proliferation of A549 cells.

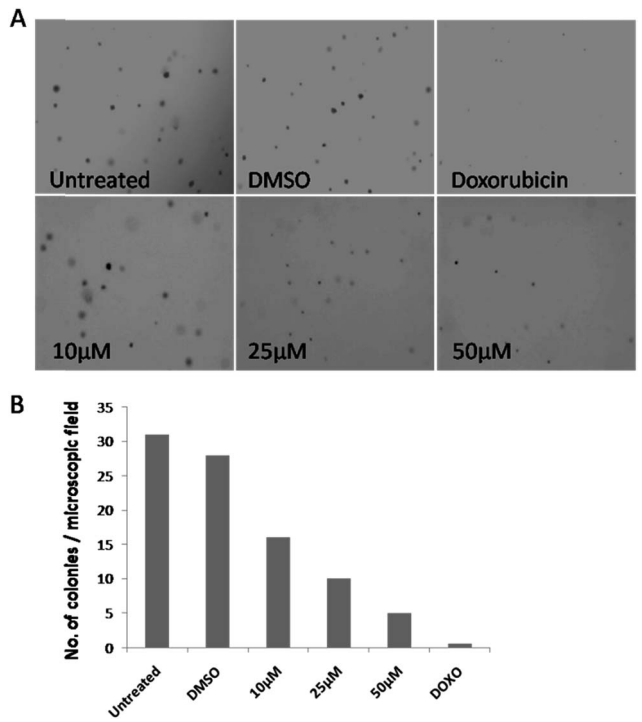


Fig. 5 Long-term effect of **NSC 771432** on the number of colony-forming A549 cells. Cells were treated with the compound (0–50 μM) and allowed to grow for 9 days to form colonies. (A) Representative images of the colony-forming assay, enlarged images show colony morphology changes induced by increasing concentration of **3a**. (B) Represents the number of colonies positively stained with crystal violet on termination of assay.

***NSC 771432** inhibits proliferation of A549 lung cancer cells.* To evaluate the inhibitory effects of **NSC 771432** on the proliferation of A549 lung epithelial cancer cells, these were treated with increasing concentrations of the compound. As shown in Fig. 4A, upon exposure of cells to the compound cells seem to lose their morphology. The compound also induced a dose dependent decrease in cell viability of the A549 cells (Fig. 4B). Further, in the time dependent experiment with A549 cells after 24 h, the anti-proliferative activity of **NSC 771432** increased by ~2-fold thereby a significant decrease in proliferation was observed after 24 h of treatment of cells of 10 μM (Fig. 4C).

To determine the long-term effect of **NSC 771432** on anchorage independent growth A549 cells, a soft agar based clonogenic assay was performed. The results obtained have clearly shown that **NSC 771432** inhibits the formation of colonies by A549 cells. From the results, a dose dependent inhibition was also observed on treatment of cells with **NSC 771432** indicating the high efficacy of **NSC 771432** towards inhibition of A549 cell growth (Fig. 5A and B), 14 days of incubation, the colonies observed in plates were stained with 0.005% crystal violet solution. After removing excess staining solution, colonies were observed and counted under a light microscope. Each experiment was performed in triplicates and repeated 3 times. The results analyzed are from three independent experiments each in triplicates. From the results presented, it is clearly evident that a significant dose-dependent inhibition of proliferation and complete change in the cell morphology and clonogenicity were observed in lung cancer cells tested. **NSC 771432** showed that significant reduction in colony formation

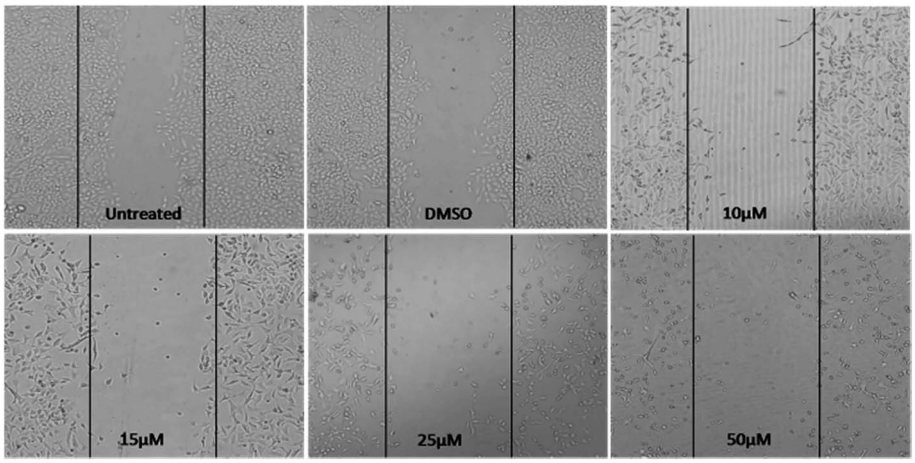


Fig. 6 Effect of NSC 771432 on A549 cell migration. Representative images from phase-contrast microscopy of cells migrating into the scratch area with or without the compound by an *in vitro* scratch assay are shown here. 24 h after wounding of the scratches created for A549 cells incubated with DMSO and the compound at different concentrations (0–50 μM) were analyzed with a phase-contrast microscope and images are presented. NSC 771432 inhibited migration of A549 cells in a concentration dependent manner.

and change in the colony morphology confirm suppressed anchorage independent growth significantly.

***NSC 771432** migration of A549 lung cancer cells.* Inhibition of cell migration is also an important property of the compounds in the anti-cancer drug discovery pipeline. Scratch assays are the best models of wound healing for epithelial or mesenchymal cells.[19] In the present study as shown in Fig. 4, the results clearly confirm that **NSC771432** inhibited migration of cells by 50% approximately even at the lowest tested concentration whereas in DMSO plates cells were migrated as same as control untreated assays (Fig. 6). The inhibitory effect is amplified by increasing the concentration of the compound and Fig. 6 shows the representation of the three experiments each performed in triplicates.

***NSC 771432** induces growth arrest of A549 cells through senescence.* Thus, after observing the effect of **NSC 771432** on proliferation and migration of A549 cells, we next studied whether treatment of cells with the compound leads to apoptosis and/or inhibit cell proliferation. Cell death and cell cycle analyses were performed using a flow cytometer. After treatment of A549 cells with the compound for 48 h, propidium iodide (PI) was applied to measure cell cycle progression and cell death. Based on the results, we observed an increase in the fraction of cells in the SubG1 phase, indicating cell death upon treatment with compound NSC 771432 (Fig. 7A–D and Table 1). To obtain further insight into the mechanisms by which **NSC 771432** treatment induces cell death and inhibit proliferation, we have investigated caspase activation and senescence in A549 cells treated with the compound. A significant increase in the caspase activity could not be observed in compound treated cells compared to control (data not shown). Therefore, we assessed the endogenous level of β-galactosidase as an indicator for senescence in A549 cells upon treatment with **NSC 771432.** Interestingly, we could observe more cells with an increased level of SA-β-gal in cells upon treatment with the compound and the number of cells positive for SA-β-gal also increased with

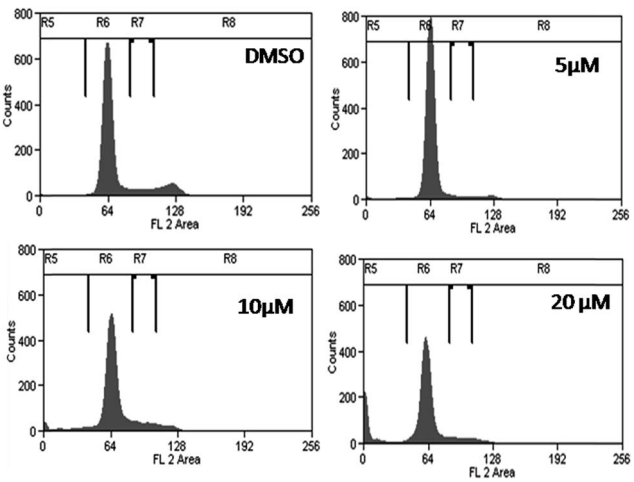


Fig. 7 NSC 771432 induces arrest of the A549 cell G2/M phase measured by the DNA content using flow cytometry.

increasing concentration. These results support the hypothesis that the exposure of cells to compound **NSC 771432** leads to the induction of cellular senescence in A549 lung cancer cells (Fig. 8) for cell cycle arrest.

Table 1 NSC 771432 induced G0/G1 phase cell cycle arrest in A549 cells. Cells were treated with varying concentrations of 044 (5, 10 and 20 μM) for 48 h and cell cycle progression was examined by flow cytometry. The table shows the percentage of cell fractions in G0/G1, S and G2/M phases of NSC 771432 treated A549 cells

	Sub G1	G0/G1	S	G2/M
DMSO	0.96	79.34	6.67	13.02
5 μM	2.49	89.38	3.47	4.65
10 μM	7.63	75.7	9.95	6.73
20 μM	19.34	69.52	6.63	4.51

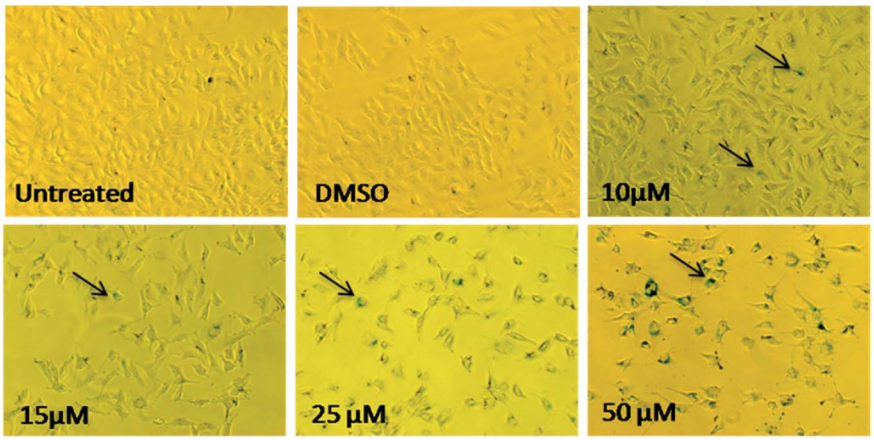


Fig. 8 Senescence induced by NSC 771432 was quantified using SA-β-gal-staining. SA-β-gal positive cells are indicated with arrow marks. The number of SA-β-gal positive cells is increased with increasing concentration of the compound indicating that NSC 771432 induced senescence of A549 cells.

3. Conclusions

A series of 2,4,5-trisubstituted and 1,2,4,5-tetrasubstituted imidazole derivatives were synthesized by multi-component reaction and their anti-cancer potential was evaluated against the panel of NCI 60 cancer cell lines. **NSC 771432** displayed significant cytotoxic potential across the NCI 60 cell line panel. Further, *in vitro* studies confirmed that the **NSC 771432** affects proliferation, migration, and anchorage independent growth of lung cancer cells by inducing cellular senescence. Taken together, the current study significantly highlights the potential of imidazole based molecules to develop novel anti-cancer agents.

4. Experimental protocols

4.1. General

Freshly distilled solvents dried over standard drying agents were used. Chemicals were purchased and used without further purification. Column chromatographic separations were carried out using silica gel (60–120 mesh). Organic solutions were dried over anhydrous Na_2SO_4 and concentrated below 40 °C *in vacuo*. ^{1}H NMR spectra (300 MHz, 500 MHz and 600 MHz) and ^{13}C NMR spectra (75 MHz and 125 MHz) with TMS as an internal standard for solutions in $CDCl_3$ were acquired on a Bruker Avance 300 MHz, an Innova 500 MHz and a Bruker 600 MHz instruments. *J* values were given in Hz. IR-spectra were recorded on a FT IR (Perkin-Elmer IR-683) spectrophotometer with NaCl optics. Mass spectra were recorded on a direct inlet system or LC by MSD trap SL (Agilent Technologies), and the HRMS data were obtained using Q-TOF mass spectrometry.

4.2. 1-Cyclohexyl-2-(4-(cyclopentyloxy)-3-methoxyphenyl)-4,5-diphenyl-1*H*-imidazole (3)

A solution of 4-(cyclopentyloxy)-3-methoxybenzaldehyde **2a** (0.20 g, 0.9 mmol), benzil (0.19 g, 0.9 mmol), cyclohexyl amine (0.10 g, 1.02 mmol) and ammonium acetate (0.7 g, 0.9 mmol) in acetic acid (2 mL) was heated at 110 °C for 24 h. The reaction mixture was quenched with solid $NaHCO_3$ (3 g) and extracted with ethyl acetate (2 × 20 mL). The organic layer was washed with water (20 mL) and brine, (15 mL) and dried (Na_2SO_4). The solvent was evaporated under reduced pressure and purified the residue by column chromatography (60–120 mesh silica gel, 11% ethyl acetate in petroleum ether) to afford **3** (0.29 g, 65%) as a yellow solid (m.p. 154–156 °C); IR (neat): 3058, 2927, 2853, 2313, 1668, 1604, 1580, 1487, 1446, 1418, 1358, 1328, 1258, 1221, 1137, 1032, 983, 818, 773, 700 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ 7.45 (m, 7H, ArH), 7.15–7.05 (m, 5H, –ArH), 6.96 (d, 1H, J = 8.2 Hz, –ArH), 4.85 (m, 1H, OCH), 3.99 (tt, 1H, J = 3.2, 12.2 Hz, CH–N), 1.96 (m, 3H, –CH_2), 1.85 (m, 4H, CH_2), 1.76–1.52 (m, 10H, –CH), 1.45 (m, 1H, –CH); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): δ 149.6, 132.2, 128.7, 129.1, 128.9, 127.9, 127.8, 126.8, 122.6, 122.2, 114.2, 114.0, 80.5, 56.1, 33.5, 32.9, 32.6, 29.7, 25.0, 26.4, 26.2, 24.1, 24.0; HRMS (ESI) *m*/*z* calculated for $C_{33}H_{37}N_2O_2$ $(M + H)^+$ 493.2850, found 493.2827.

4.3. 2-(4-(Cyclopentyloxy)-3-methoxyphenyl)-4,5-diphenyl-1*H*-imidazole (4)

A solution of 4-(cyclopentyloxy)-3-methoxybenzaldehyde **2a** (0.20 g, 0.9 mmol), benzil (0.19 g, 0.9 mmol) and ammonium acetate (0.20 g, 1.8 mmol) in acetic acid (2 mL) was heated at 110 °C for 24 h. Workup as described for **3** and purification of the residue by column chromatography (60–120 mesh silica gel, 11% ethyl acetate in petroleum ether) afforded **4** (0.26 g, 68%) as a pale yellow solid (m.p. 198–200 °C); IR (neat): 3308, 3060, 2957, 2924, 2853, 1719, 1665, 1604, 1493, 1451, 1379, 1326, 1257, 1225, 1032, 982, 761, 696 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ 7.56 (s, 1H, –NH), 7.79 (m, 4H, –ArH), 7.39 (d, 1H, J = 8.1 Hz, –ArH), 7.27 (m, 7H, –ArH), 6.83 (d, 1H, J = 8.1 Hz, –ArH), 4.74 (m, 1H, –OCH), 3.75 (s, 3H, OCH_3), 1.92–1.77 (m, 8H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): δ 149.9, 148.5, 146.2, 132.2, 128.4, 127.8, 127.3, 121.9, 118.0, 114.1, 109.4, 80.33, 55.8, 32.9, 29.7, 24.1; HRMS (ESI) *m*/*z* calculated for $C_{27}H_{27}N_2O_2$ $(M + H)^+$ 411.2067, found 411.2061.

4.4. 2-(4-(Cyclopentyloxy)-3-methoxyphenyl)-4,5-diphenyl-1-(prop-2-ynyl)-1*H*-imidazole (5)

A solution of 4-(cyclopentyloxy)-3-methoxybenzaldehyde **2a** (0.20 g, 0.9 mmol), benzil (0.28 g, 0.9 mmol), propargyl amine (0.11 mL, 1.0 mmol) and ammonium acetate (0.11 g, 0.9 mmol) in acetic acid (2 mL) was heated at 85 °C for 24 h. Workup as described for **3** and purification of the residue by column chromatography (60–120 mesh silica gel, 10% ethyl acetate in petroleum ether) afforded **5** (0.26 g, 65%) as a pale yellow solid (m.p. 192–194 °C); IR (neat): 3311, 3008, 2955, 2921, 2852, 1744, 1605, 1583, 1494, 1461, 1376, 1362, 1258, 1219, 1166, 1119, 1075, 1032, 983, 912, 853, 812, 696, 687 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 298 K): δ 7.54 (m, 6H, –ArH), 7.31 (m, 6H, –ArH), 6.89 (d, 1H, $J = 8.9$ Hz, –ArH), 5.35 (d, 2H, $J = 5.2$ Hz, N–CH_2), 4.80 (m, 1H, OCH), 3.88 (s, 3H, –OCH_3), 2.77 (t, 1H, $J = 5.2$ Hz, ≡CH), 2.08–1.81 (m, 8H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): δ 150.2, 148.6, 148.5, 146.1, 131.1, 130.2, 130.0, 128.5, 127.9, 127.4, 126.0, 117.8, 114.3, 109.6, 80.5, 56.2, 32.8, 29.7, 29.6, 24.1; HRMS (ESI) *m*/*z* calculated for $C_{30}H_{29}N_2O_2$ $(M + H)^+$ 449.2223, found 449.2208.

4.5. 2-(3-(Cyclopentyloxy)-4-methoxyphenyl)-1-hexyl-4,5-diphenyl-1*H*-imidazole (6)

A solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde **2b** (0.20 g, 0.9 mmol), benzil (0.19 g, 0.9 mmol), *n*-hexyl amine (0.11 g, 1.09 mmol) and ammonium acetate (0.06 g, 0.9 mmol) in acetic acid (2 mL) was heated at 110 °C for 24 h. Workup as described for **3** and purification of the residue by column chromatography (60–120 mesh silica gel, 6% ethyl acetate in petroleum ether) afforded **6** (0.19 g, 67%) as a pale yellow solid (m.p. 93–94 °C); IR (neat): 3058, 2955, 2927, 2855, 1603, 1582, 1528, 1484, 1463, 1442, 1360, 1321, 1261, 1221, 1173, 1137, 1072, 1052, 986, 904, 863, 812, 774, 755, 699 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 298 K): δ 7.46 (m, 7H, –ArH), 7.18 (m, 5H, –ArH), 6.96 (d, 1H, $J = 8.9$ Hz, –ArH), 4.86 (m, 1H, –OCH), 3.91 (s, 3H, –OCH_3), 3.85 (t, 2H, $J = 7.7$ Hz, –CH_2–N), 1.88 (m, 8H, –CH), 1.62 (m, 2H, –CH), 1.33 (m, 2H, –CH), 1.05 (m, 1H, –CH), 0.95 (m, 3H, –CH), 0.74 (t, 3H, $J = 7.0$ Hz, –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): δ 150.63, 147.6, 147.4, 137.2, 134.5, 130.9, 129.3, 129.0, 128.5, 127.9, 126.7, 126.1, 123.9, 121.9, 115.9, 111.6, 80.5, 56.0, 44.7, 32.8, 30.8, 30.3, 25.9, 24.1, 22.2, 13.8; HRMS (ESI) *m*/*z* calculated for $C_{33}H_{39}N_2O_2$ $(M + H)^+$ 495.3006, found 495.2987.

4.6. 2-(3-(Cyclopentyloxy)-4-methoxyphenyl)-4,5-diphenyl-1*H*-imidazole (7)

A solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde **2b** (0.2 g, 0.9 mmol), benzil (0.19 g, 0.9 mmol) ammonium acetate (0.2 g, 1.80 mmol) in acetic acid (5 mL) was heated at 110 °C for 24 h. Workup as described for **3** and purification of the residue by column chromatography (60–120 mesh silica gel, 11% ethyl acetate in petroleum ether) afforded **7** (0.17 g, 69%) as a pale yellow solid (m.p. 225–227 °C); IR (neat): 3747, 3309, 3059, 2959, 2869, 2314, 1658, 1605, 1539, 1494, 1451, 1434, 1408, 1360, 1326, 1256, 1222, 1168, 1118, 1032, 983, 913, 852, 818, 769 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 298 K): δ 8.44 (br. s, 1H, –NH), 7.64 (m, 4H, –ArH), 7.38 (m, 8H, –ArH), 7.0 (d, 1H, $J = 8.5$ Hz, –ArH), 4.90 (m, 1H, –OCH), 4.0 (s, 3H, –OCH_3), 1.99 (m, 5H, –CH_2), 1.71 (m, 3H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): 150.1, 148.4, 146.4, 128.5, 127.8, 127.7, 127.2, 117.6, 114.4, 109.5, 80.5, 55.9, 32.8, 24.1; HRMS (ESI) *m*/*z* calculated for $C_{27}H_{27}N_2O_2$ $(M + H)^+$ 411.2067, found 411.2059.

4.7. 2-(3-(Cyclopentyloxy)-4-methoxyphenyl)-4,5-diphenyl-1-(prop-2-ynyl)-1*H*-imidazole (8)

A solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde **2b** (0.2 g, 0.9 mmol), benzil (0.19 g, 0.9 mmol), ammonium acetate (0.13 g, 0.9 mmol) and propargyl amine (0.14 mL, 0.14 mmol) in acetic acid (5 mL) was heated at 85 °C for 24 h. Workup as described for **3** and purification of the residue by column chromatography (60–120 mesh silica gel, 17% ethyl acetate in petroleum ether) afforded **8** (0.35 g, 68%) as a pale yellow solid (m.p. 160 °C); IR (neat): 3745, 3280, 3006, 2958, 2869, 2313, 2120, 1678, 1603, 1531, 1487, 1445, 1346, 1322, 1263, 1217, 1177, 1137, 1026, 942, 772, 698, 634 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 298 K): δ 7.52 (m, 9H, –ArH), 7.19 (m, 3H, –ArH), 6.99 (d, 1H, $J = 8.6$ Hz, –ArH), 4.92 (m, 1H, –OCH), 4.48 (d, 2H, $J = 2.26$ Hz, –CH_2–N), 3.91 (s, 3H, –OCH_3), 2.45 (t, 1H, $J = 2.26$ Hz, ≡CH), 2.05–1.80 (m, 8H, –CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): δ 150.7, 147.8, 147.5, 134.3, 131.1, 130.6, 129.6, 129.0, 128.8, 128.0, 126.9, 126.4, 122.7, 121.7, 115.1, 111.7, 80.4, 79.7, 73.5, 55.8, 35.3, 32.8, 24.4; HRMS (ESI) *m*/*z* calculated for $C_{30}H_{29}N_2O_2$ $(M + H)^+$ 449.2223, found 449.2203.

4.8. 2-(3-(Cyclopentyloxy)-4-methoxyphenyl)-1-cyclopropyl-4,5-diphenyl-1*H*-imidazole (9)

A solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde **2b** (0.20 g, 0.9 mmol), benzil (0.19 g, 0.9 mmol), cyclopropyl amine (0.08 g, 1.2 mmol) and ammonium acetate (0.13 g, 0.9 mmol) in acetic acid (2 mL) was heated at 50 °C for 24 h. Workup as described for **3** and purification of the residue by column chromatography (60–120 mesh silica gel, 6% ethyl acetate in petroleum ether) afforded **9** (0.24 g, 61%) as a pale yellow solid (m.p. 183–185 °C); IR (neat): 3059, 3010, 2957, 2870, 1693, 1603, 1583, 1527, 1483, 1445, 1368, 1320, 1256, 1219, 1167, 1130, 1023, 985, 751, 697 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 298 K): δ 7.55 (m, 2H, –ArH), 7.42 (m, 7H, –ArH), 7.22 (m, 2H, –ArH), 7.14 (m, 1H, –ArH), 6.95 (d, 1H, $J = 8.85$ Hz, –ArH), 4.89 (m, 1H, –OCH), 3.91 (s, 3H, OCH_3), 3.32 (m, 1H, –CH–N), 1.97 (m, 4H, –CH), 1.87 (m, 2H, CH), 1.62 (m, 2H, –OCH), 0.67 (m, 2H, –CH_2), 0.39 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): δ 150.4, 148.9, 147.1, 136.9, 134.6, 131.4, 130.4, 128.5, 128.0, 127.8, 127.3, 126.3, 123.8, 121.8, 115.7, 111.3, 80.5, 55.9, 32.9, 28.1, 24.1, 10.4; HRMS (ESI) *m*/*z* calculated for $C_{30}H_{31}N_2O_2$ $(M + H)^+$ 451.2380, found 451.2364.

4.9. 2,2′-(2-(3-(Cyclopentyloxy)-4-methoxyphenyl)-1-isobutyl-1*H*-imidazole-4,5-diyl)dipyridine (10)

A solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde **2b** (0.15 g, 0.70 mmol), pyridil (0.18 g, 0.85 mmol), isobutyl amine (0.14 mL, 0.47 mmol) and ammonium acetate (0.03 g, 0.04 mmol) in acetic acid (5 mL) was heated at 95 °C for 24 h.

Workup as described for **3** and purification of the residue by column chromatography (60–120 mesh silica gel, 11% ethyl acetate in petroleum ether) afforded **10** (0.17 g, 55%) as a pale yellow solid (m.p. 180–182 °C); IR (neat): 2958, 2929, 2871, 1587, 1532, 1488, 1469, 1438, 1388, 1362, 1324, 1263, 1247, 1217, 1137, 1090, 1026, 978, 743, 664, 601 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 298 K): δ 8.74 (d, 1H, J = 4.6 Hz, –PyH), 8.46 (d, 1H, J = 4.2 Hz, –ArH), 7.71–7.56 (m, 4H, –PyH), 7.28–7.19 (m, 3H, –ArH), 7.08 (m, 1H, –ArH), 6.96 (d, 1H, J = 7.9 Hz, –ArH), 4.85 (m, 1H, OCH), 4.13 (d, 2H, J = 7.5 Hz, $-CH_2-N$), 3.90 (s, 3H, $-OCH_3$), 1.97–1.80 (m, 7H, CH_2), 1.62 (m, 2H, CH_2), 0.50 (d, 6H, J = 7.7 Hz, $(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): 153.7, 151.1, 150.5, 149.8, 149.2, 148.9, 147.3, 135.9, 130.2, 127.4, 123.7, 122.4, 122.2, 121.4, 116.0, 111.5, 80.3, 55.9, 52.4, 32.7, 29.6, 29.1, 24.0, 19.5; HRMS (ESI) m/z calculated for $C_{29}H_{33}N_4O_2$ $(M + H)^+$ 469.2598, found 469.2584.

4.10. 2-(3-(Cyclohexyloxy)-4-methoxyphenyl)-1-cyclopropyl-4,5-diphenyl-1*H*-imidazole (11)

A solution of 3-(cyclohexyloxy)-4-methoxybenzaldehyde **2d** (0.2 g, 0.85 mmol), benzil (0.18 g, 0.85 mmol), ammonium acetate (0.07 g, 0.85 mmol) and cyclopropyl amine (0.06 g, 1.02 mmol) in acetic acid (5 mL) was heated at 120 °C for 24 h. Workup as described for **3** and purification of the residue by column chromatography (60–120 mesh silica gel, 10% ethyl acetate in petroleum ether) afforded **11** (0.24 g, 62%) as a pale yellow solid (m.p. 182 °C); IR (neat): 3011, 2927, 2854, 1663, 1603, 1582, 1526, 1482, 1448, 1369, 1259, 1218, 1170, 1128, 1074, 1025, 960, 875, 812, 665 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 298 K): δ 7.54 (m, 2H, –ArH), 7.46–7.37 (m, 7H, –ArH), 7.22 (m, 2H, –ArH), 7.15 (m, 1H, –ArH), 6.96 (d, 1H, J = 8.4 Hz, –ArH), 4.32 (m, 1H, OCH), 3.91 (s, 3H, $-OCH_3$), 3.31(sept, 1H, J = 3.6, –CH–N), 2.08 (m, 2H, –CH), 1.84 (m, 2H, CH), 1.60 (m, 3H, –CH), 1.41–1.26 (m, 3H, –CH), 0.68 (q, 2H, J = 7.2, $-CH_2$), 0.39 (m, 2H, $-CH_2$); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): 151.0, 148.8, 146.5, 136.9, 134.6, 131.6, 131.5, 130.4, 128.5, 128.0, 127.8, 127.3, 126.3, 124.0, 122.3, 117.3, 111.4, 77.8, 55.91, 32.1, 28.1, 24.1, 10.3; HRMS (ESI) m/z calculated for $C_{31}H_{33}N_2O_2$ $(M + H)^+$ 465.2536, found 465.2519.

4.11. 1-Benzyl-2-(4-(cyclohexyloxy)-3-methoxyphenyl)-4,5-diphenyl-1*H*-imidazole (12)

A solution of 4-(cyclohexyloxy)-3-methoxybenzaldehyde **2d** (0.20 g, 0.85 mmol), benzil (0.18 g, 0.85 mmol), benzyl amine (0.10 g, 1.02 mmol) and ammonium acetate (0.06 g, 0.88 mmol) in acetic acid (2 mL) was heated at 110 °C for 24 h. Workup as described for **3** and purification of the residue by column chromatography (60–120 mesh silica gel, 11% ethyl acetate in petroleum ether) afforded **12** (0.25 g, 63%) as a pale yellow solid (m.p. 119–121 °C); IR (neat): 3743, 3610, 3061, 3030, 2853, 1740, 1676, 1603, 1580, 1525, 1489, 1449, 1420, 1358, 1312, 1267, 1218, 1181, 1146, 1025, 964, 864, 770, 729, 698 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 298 K): δ 7.57 (m, 2H, –ArH), 7.33 (m, 3H, –ArH), 7.22 (m, 7H, –ArH), 7.13 (m, 3H, –ArH), 6.89 (m, 3H, –ArH), 5.09 (s, 2H, $-CH_2Ar$), 4.21 (m, 1H, –OCH), 3.65 (s, 3H, OCH_3), 2.02 (m, 3H, CH_2), 1.81 (m, 3H, CH_2), 1.57 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz, 298 K): δ 150.0, 148.1, 147.7, 137.6, 134.3, 131.0, 129.9, 128.7, 128.6, 128.0, 127.3, 126.8, 126.3, 125.9, 123.9, 123.4, 121.4, 115.4, 112.8, 55.6, 48.2, 31.8, 31.4, 30.1, 29.6, 25.6, 24.0; HRMS (ESI) m/z calculated for $C_{35}H_{35}N_2O_2$ $(M + H)^+$ 515.2693, found 515.2677.

5. Cell culture

The A549 cell lines used in the present study were obtained from ATCC (Manassas, USA) and cultivated in DMEM (Sigma) supplemented with 10% fetal bovine serum, 100 units per mL penicillin and streptomycin. To avoid contamination of cells with mycoplasma, cells were regularly tested with the MycoAlert Kit (Cambrex Bio Science, Rockland, ME, USA).

5.1. Cytotoxicity evaluation against A549 cells

Cell viability was determined by MTT-micro-cultured tetrazolium assay using the reported protocol with minor modifications.[20] Briefly, A549 cells were seeded into the flat bottom 96-well plate (5000 cells per 100 μL) and cultured for 18–24 h in the culture medium with a constant supply of 5% CO_2 in a humid incubator. The different concentrations of the test compound and doxorubicin (as standard control anti-cancer drug) prepared in DMSO were added to achieve the final concentration of 0 to 100 μM of the compound to cells and cells were further continued to grow for 48 h with a constant supply of 5% CO_2 in a humid incubator. The time course (0 to 72 h) experiment was carried out simultaneously. Filter sterilized MTT dissolved in PBS at 5 mg mL^{-1} was added for assay at indicated time intervals. Cells were further incubated in the CO_2 chamber for 2 h. On termination of assay, the medium was removed and 100 μL of DMSO was added to cells. The purple colour obtained was measured at 562 nm using a multimode microplate reader (Tecan GENios); its absorbance is directly proportional to cell growth. From the observed percentage age of growth with and without the test compound, IC_{50} values were calculated. The results presented are from three independent experiments each in triplicates.

5.2. Colony formation assay

For observing long term effects of **NSC 771432** on anchorage-independent growth of A549 cells soft agar assays were performed as reported previously.[21] To perform soft agar assay, bottom agar was prepared by mixing 1% of agarose (Bacto Agar: Becton, Dickinson, Sparks, MD) with 2× culture medium poured into 6-well plates at 37 °C to achieve a final concentration of 0.5% of agar in 1× growth medium. Once bottom agar was solidified, 2.5×10^4 cells were mixed with cultivation medium containing different concentrations of the compound and equal volume of agar solution achieving a final concentration of 0.35% agar. The mixture was spread on the surface of base agar plates immediately and incubated under cultivation conditions. To avoid depletion of nutrients, culture medium was replenished every 3 days with fresh medium along with the compound. After 14 days of incubation, the colonies observed in plates were stained with 0.005% crystal violet solution. After removing excess staining solution, colonies were observed and

counted under a light microscope. Each experiment was performed in triplicates and repeated 3 times. The results analyzed are from three independent experiments each in triplicates.

5.3. Wound healing/cell migration assay

Cell migration assays were performed, as reported previously, with minor modifications. Briefly, after harvesting sub-confluent A549 cells by trypsinization the pellet was resuspended in culture medium by gently pipetting to obtain single cells. For assays, 5×10^4 cells per well seeded into 24-well plates and allowed to grow for 18–24 h under standard culture conditions to create a confluent monolayer of cells. To determine the effect of the compound on migration of cells, scraping the cell monolayer with a p200 pipette tip creates "scratches". After smoothening the edge of the scratch by removing debris by washing with 0.5 mL of growth medium the medium was replaced with 250 μL of medium containing a defined concentration of compound or DMSO as vehicle control. Further, cells were incubated to grow for 24 h allowing migration of cells closing the scratches created in the dishes. The images acquired for each sample under a phase-contrast microscope were analyzed to observe influence of the compound on migration of cells compared to DMSO treated control. By comparing the images from initial time point 0, results were interpreted as presented in Fig. 6. Three individual experiments were performed in triplicates; only representative images are presented here.

5.4. Senescence associated β-gal staining

Detection of SA-β-galactosidase was performed as described previously.[20] Briefly, A549 cells treated with different concentrations of the compound or DMSO were harvested at sub-confluent density and fixed in phosphate buffered saline containing 2% PFA and 0.25% glutaraldehyde along with 1 mM $MgCl_2$ (pH 6.0). Then the fixed cells were incubated in a staining solution containing potassium cyanide/X-gal in PBS/$MgCl_2$ (pH 6.0) at 37 °C overnight. Slides were analyzed using a Xi72 microscope (10 Å ~ magnifications) (Olympus, Japan). The positive cells for β-gal staining upon exposure to the compound are marked on images.

5.5. PI uptake for cell death

Cell death was analyzed with PI uptake as reported previously with minor modification.[19] Cells were harvested, washed with PBS and fixed in 70% ethanol at −20 °C overnight. After centrifugation, cells were resuspended in PI solution containing RNase (0.1 mg mL^{-1}), Triton X-100 (0.05%), and PI (50 μg mL^{-1}) and incubated for 1 h in the dark at room temperature. After washing with PBS buffer, PI uptake in cells was analyzed by fluorescence activated cell sorting (FACS Calibur System; BD Bio-science, Erembodegem, Belgium) on an FL-2 fluorescence detector (10 000 events were recorded for each condition). Flow cytometry data were analyzed using FCS express 4 software (De Novo Software, Los Angeles, CA).

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