New pyridine and chromene scaffolds as potent vasorelaxant and anticancer agents

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Fig. 1: MTT is reduced by NADPH to form purple formazan crystals

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Spectroscopic chart captions

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1. Vasodilation Activity Screening

The vasodilation activity screening was undertaken by Pharmacology Department, National Research Centre, Egypt, according to the standard in vitro bioassay technique [1-4] by testing the effects of the synthesized agents 3a-o and 4a-e and compared with prazosin hydrochloride (α1-AR antagonist) on isolated thoracic aortic rings of male Wistar rats (200–250 g) pre-contracted with norepinephrine hydrochloride. After light ether anesthesia, the rats were sacrificed by cervical dislocation. The aortae were immediately excised, freed of extraneous tissues, and prepared for isometric tension recording. Aorta was cut into (3-5 mm width) rings and each ring was placed in a vertical chamber “10 ml 5 jacketed automatic multi-chamber organ bath system (Model no. ML870B6/C, Panlab, Spain)” filled with Krebs solution composed of (in mM): NaCl, 118.0; KCl, 4.7; NaHCO₃, 25.0; CaCl₂, 1.8; NaH₂PO₄, 1.2; MgSO₄, 1.2; glucose, 11.0 and oxygenated with carbogen gas (95% O₂/5% CO₂) at 37 ± 0.5 °C. Each aortic ring was mounted between two stainless steel hooks passed through its lumen. The lower hook was fixed between two plates, while the upper one was attached to a force displacement transducer (Model no. MLT0201, Panlab, Spain) connected to an amplifier (PowerLab, AD Instruments Pty. Ltd.), which was connected to a computer. The Chart for windows (v 3.4) software was used to record and elaborate data. Preparations were stabilized under 2 g resting tension during 2 h, and then the contracture response to norepinephrine hydrochloride (10-6 M) was measured before and after exposure to increasing concentrations of the tested synthesized compounds (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µM). The tested compounds were dissolved in dimethyl sulfoxide
(DMSO) as stock solution (10 ml of 0.005 M). Control experiments were performed in the presence of DMSO alone, at the same concentrations as those used with the derivatives tested, which demonstrated that the solvent did not affect the contractile response of isolated aorta. The observed vasodilation activity screening data for the synthesized compounds 3a-o and 4a-e and prazosin hydrochloride are expressed as IC$_{50}$ ($\mu$M) concentration necessary for 50% reduction of maximal norepinephrine hydrochloride induced contracture utilizing four different replicates.

2. Cell Culture Methods

Cell culturing
The MCF-7 cells were cultured in RPMI with 5% FBS. The MDA-MB 231 cells was cultured in DMEM (with L-glutamine) with 10% FBS. All of the cells were stored in an incubator at 37 °C with 5% CO$_2$. The cells were subcultured at 80-90 % confluency. For MCF-7 and MDA-MB 231, the old medium was removed and discarded, and the flask was washed with 5 ml, phosphate buffer solution (PBS). 1 mL of trypsin was then added to the flask and allowed to incubate for 3 min before 4 mL of complete medium was added to deactivate the trypsin. The solution was then removed from the flask and put into a falcon tube and the cells were separated from the medium by centrifuging at 215 x g for 5 min. The old medium was removed and discarded, and fresh medium was added and the cells suspended, and new flasks were seeded at 1:10 cells to complete medium.

Cell counting
To count the cells, the same procedure for detaching cells as subculturing was used. After the cells were resuspended in fresh medium, a 100 $\mu$L aliquot was added to 100 $\mu$L of Trypan blue (TB) solution. The cells were then counted using a haemocytometer. The haemocytometer has a neubauer chamber, which contains two sets of 9 squares in a grid. Using the haemocytometer, 5 squares from each set were counted and all 10 squares averaged, and the concentration of cells in the solution was calculated using the following equation:
Concentration (cells/mL) = 2 x Cells/square average x 10^4

In this equation, 2 is the dilution factor from the addition of the TB solution and 10^4 is the converted value of the volume each square contains. Using the concentration of cells that was determined, an aliquot of the cell suspension was diluted with fresh medium to the desired density for seeding a 96 well plate. Plates were seeded 24 hours before they were treated with the test compounds.

**Phosphate Buffer Saline Preparation**

Phosphate buffer saline (PBS) was prepared by adding 4.4 g NaCl, 7.5 g Na₂HPO₄, and 2.1 g Na₂HPO₄ to a 1 L media bottle containing approximately 750 mL deionized water and stirred until completely dissolved. The pH was checked to confirm it was 7.4, and then the volume was brought up to 1 L with deionized water. The buffer was then decanted into smaller bottles before autoclaving.

**Test compounds stock solutions**

The compounds were provided in powdered form, and to assess the compounds' activities against various cell lines, stock solutions were prepared by dissolving them in DMSO to a concentration of 4 mM. The stock solutions were stored in the freezer at -20 °C.

**MTT cell viability assay**

Cytotoxicity of the compounds was tested using the MTT assay. MTT (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide) is a salt that is enzymatically reduced by live cells, resulting in the formation of formazan crystals which can then be read by a spectrophotometer.

To test the compounds against MCF-7 and NIDA-MB 231 cells, solutions were prepared from the 4 mM stock solutions. They were diluted to 20 μM in complete medium to contain 1% DMSO. MCF-7 and MDA-MB 231 were screened against all compounds at a concentration of 10 μM by adding 100 μL of the diluted solution to each well. The four compounds that exhibited the highest cytotoxic effect were selected to find the IC₅₀ for each of the cancer cell lines in the MCF-7 and MDA-MB 231 experiments were selected. Each experiment was replicated three times, and the results were expressed as the mean ± the standard error of the mean.

After the initial screening, the selected compounds were again plated and screened at concentrations ranging from 0.01 μM to 20 μM, and the MTT assay was carried out
again. Each experiment was replicated three times, and the results were expressed as the mean ± the standard error of the mean. The results were plotted in graph pad in order to determine the IC$_{50}$ values.

![Chemical Structure]

**Fig. 1:** MTT is reduced by NADPH to form purple formazan crystals

An MTT solution was prepared by dissolving MTT in PBS at a concentration of 5 mg/mL. 20 µL of the solution was added to each of the wells treated with a compound, as well as to the control, and allowed to incubate for 5 h. After 5 h, the media in the wells was removed and the formazan crystals were dissolved in 100 µL of DMSO. The plate was then incubated for 30 min to allow the formazan crystals to dissolve, and the plate was read on a spectrophotometer at 570 nm, with a reference wavelength of 450 nm. Each experiment was replicated three times, and the results were expressed as the mean ± the standard error of the mean.
Table S1. Dose response curves the tested compounds 3a-o and 4a-e.

| Compound | IC50 (µM) | Concentration (µM) | NE.HCl contracture (control, %) |
|----------|-----------|--------------------|----------------------------------|
| 3a       | 437.9     | 0-800              | 0-100                            |
| 3b       | 489.7     | 0-800              | 0-100                            |
| 3c       | 532.8     | 0-800              | 0-100                            |
| 3d       | 524.1     | 0-800              | 0-100                            |
3e, IC50 = 558.6 µM

3f, IC50 = 608.6 µM

3g, IC50 = 698.3 µM

3h, IC50 = 481.0 µM

3i, IC50 = 758.6 µM

3j, IC50 = 484.5 µM
3k, IC50 = 584.5 µM

3l, IC50 = 558.6 µM

3m, IC50 = 444.8 µM

3n, IC50 = 739.7 µM

3o, IC50 = 312.1 µM

4a, IC50 = 710.2 µM
**4b, IC50 = 674.1 µM**

**4c, IC50 = 639.7 µM**

**4d, IC50 = 427.6 µM**

**4e, IC50 = 417.2 µM**

Concentration (µM) vs. NE.HCl contracture (control, %) for compounds 4b, 4c, 4d, and 4e.
Fig. 2. IC\textsubscript{50} curves of compounds 3d, 3g, 3h and 3i on MCF-7 cell line

Fig. 3. IC\textsubscript{50} curves of compounds 3d, 3g and 3i on MDA-MB 231 cell line
Fig. S1. IR spectrum of compound 3a (KBr pellet).
Fig. S2. $^1$H-NMR spectrum of compound 3a.
Fig. S3. $^{13}$C-NMR spectrum of compound 3a.
Fig. S4. Mass spectrum of compound 3a.
Fig. S5. IR spectrum of compound 3b (KBr pellet).
Fig. S6. $^1$H-NMR spectrum of compound 3b.
Fig. S7. $^{13}$C-NMR spectrum of compound 3b.
Fig. S8. Mass spectrum of compound 3b.
Fig. S9. IR spectrum of compound 3c (KBr pellet).
Fig. S10. $^1$H-NMR spectrum of compound 3c.
Fig. S11. $^{13}$C-NMR spectrum of compound 3c.
Fig. S12. Mass spectrum of compound 3c.
Fig. S13. IR spectrum of compound 3d (KBr pellet).
Fig. S14. $^1$H-NMR spectrum of compound 3d.
**Fig. S15.** $^{13}$C-NMR spectrum of compound 3d.
Fig. S16. Mass spectrum of compound 3d.
Fig. S17. IR spectrum of compound 3e (KBr pellet).
Fig. S18. $^1$H-NMR spectrum of compound 3e.
Fig. S19. $^{13}$C-NMR spectrum of compound 3e.
Fig. S20. Mass spectrum of compound 3e.
Fig. S21. IR spectrum of compound 3f (KBr pellet).
Fig. S22. $^1$H-NMR spectrum of compound 3f.
Fig. S23. $^{13}$C-NMR spectrum of compound 3f.
Fig. S24. Mass spectrum of compound 3f.
Fig. S25. IR spectrum of compound 3g (KBr pellet).
Fig. S26. $^1$H-NMR spectrum of compound 3g.
Fig. S27. $^{13}$C-NMR spectrum of compound 3g.
Fig. S28. Mass spectrum of compound 3g.
Fig. S29. IR spectrum of compound 3h (KBr pellet).
Fig. S30. $^1$H-NMR spectrum of compound 3h.
Fig. S31. $^{13}$C-NMR spectrum of compound 3h.
Fig. S32. Mass spectrum of compound 3h.
Fig. S33. IR spectrum of compound 3i (KBr pellet).
Fig. S34. $^1$H-NMR spectrum of compound 3i.
Fig. S35. $^{13}$C-NMR spectrum of compound 3i.
Fig. S36. Mass spectrum of compound 3i.
Fig. S37. IR spectrum of compound 3j (KBr pellet).
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Fig. S39. $^{13}$C-NMR spectrum of compound 3j.
Fig. S40. Mass spectrum of compound 3j.
Fig. S41. IR spectrum of compound 3k (KBr pellet).
Fig. S42. $^1$H-NMR spectrum of compound 3k.
Fig. S43. $^{13}$C-NMR spectrum of compound 3k.
Fig. S44. Mass spectrum of compound 3k.
Fig. S45. IR spectrum of compound 3l (KBr pellet).
Fig. S46. $^1$H-NMR spectrum of compound 3l.
Fig. S47. $^{13}$C-NMR spectrum of compound 31.
Fig. S48. Mass spectrum of compound 3l.
Fig. S49. IR spectrum of compound 3m (KBr pellet).
Fig. S50. $^1$H-NMR spectrum of compound 3m.
Fig. S51. $^{13}$C-NMR spectrum of compound 3m.
Fig. S52. Mass spectrum of compound 3m.
**Fig. S53.** IR spectrum of compound 3n (KBr pellet).

| No. | Position [cm⁻¹] | Intensity         | No. | Position [cm⁻¹] | Intensity         | No. | Position [cm⁻¹] | Intensity         | No. | Position [cm⁻¹] | Intensity         |
|-----|-----------------|-------------------|-----|-----------------|-------------------|-----|-----------------|-------------------|-----|-----------------|-------------------|
| 1   | 3767.26         | 98.5222           | 2   | 3435.56         | 90.5107           | 3   | 3118.33         | 96.738            | 4   | 3051.8         | 96.5955           |
| 5   | 2931.27         | 77.5995           | 6   | 2856.06         | 85.8632           | 7   | 2379.73         | 96.2084           | 8   | 2304.52         | 97.0434           |
| 9   | 2221.59         | 91.6613           | 10  | 1941.97         | 97.7959           | 11  | 1906.29         | 97.8006           | 12  | 1815.65         | 97.8589           |
| 13  | 1739.48         | 90.0745           | 14  | 1560.13         | 73.4483           | 15  | 1458.89         | 69.6771           | 16  | 1376.53         | 69.6532           |
| 17  | 1302.68         | 79.6422           | 18  | 1197.94         | 79.9132           | 19  | 1128.15         | 83.6761           | 20  | 1093.44         | 82.0867           |
| 21  | 1031.73         | 88.6189           | 22  | 970.983         | 86.9092           | 23  | 846.597         | 93.3549           | 24  | 794.528         | 90.4566           |
| 25  | 748.245         | 79.6727           | 26  | 670.142         | 90.5609           | 27  | 586.254         | 92.9807           | 28  | 521.65         | 94.3747           |
Fig. S54. $^1$H-NMR spectrum of compound 3n.
Fig. S55. $^{13}$C-NMR spectrum of compound 3n.
Fig. S56. Mass spectrum of compound 3n.
Fig. S57. IR spectrum of compound 3o (KBr pellet).
Fig. S58. $^1$H-NMR spectrum of compound 3o.
Fig. S59. $^{13}$C-NMR spectrum of compound 3o.
Fig. S60. Mass spectrum of compound 3o.
Fig. S61. IR spectrum of compound 4a (KBr pellet).
Fig. S62. $^1$H-NMR spectrum of compound 4a.
Fig. S63. $^{13}$C-NMR spectrum of compound 4a.
Fig. S64. Mass spectrum of compound 4a.
Fig. S65. IR spectrum of compound 4b (KBr pellet).
Fig. S66. $^1$H-NMR spectrum of compound 4b.
Fig. S67. $^{13}$C-NMR spectrum of compound 4b.
Fig. S68. Mass spectrum of compound 4b.
Fig. S69. IR spectrum of compound 4c (KBr pellet).
Fig. S70. $^1$H-NMR spectrum of compound 4c.
Fig. S71. $^{13}$C-NMR spectrum of compound 4c.
Fig. S72. Mass spectrum of compound 4c.
Fig. S73. IR spectrum of compound 4d (KBr pellet).
Fig. S74. $^1$H-NMR spectrum of compound 4d.
Fig. S75. $^{13}$C-NMR spectrum of compound 4d.
Fig. S76. Mass spectrum of compound 4d.
Fig. S77. IR spectrum of compound 4e (KBr pellet).
Fig. S78. $^1$H-NMR spectrum of compound 4e.
Fig. S79. $^{13}$C-NMR spectrum of compound 4e.
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