Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 432–435
Bioorganic & Medicinal Chemistry Letters

Synthesis, characterization and evaluation of benzimidazole derivative and its precursors as inhibitors of MDA-MB-231 human breast cancer cell proliferation

N. R. Thimmegowda,[a] S. Nanjunda Swamy,[a,c] C. S. Ananda Kumar,[a] Y. C. Sunil Kumar,[a] S. Chandrappa,[a] George W. Yip[b,*] and K. S. Rangappa[a,*]

[a]*Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore 570006, India*
[b]*Department of Anatomy, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore*
[c]*Department of Biotechnology, Sri Jayachamarajendra College of Engineering, Mysore 570006, India*

Received 16 March 2007; revised 14 August 2007; accepted 15 August 2007
Available online 24 October 2007

Abstract—A novel series of trisubstituted benzimidazole and its precursors (**3**–**7**) were synthesised and characterized by using ^{1}H NMR, LC/MS, FTIR and elemental analysis techniques. The title compounds were evaluated for inhibition against MDA-MB-231 breast cancer cell proliferation. The results revealed that the compound *N*-(4-cyano-3-(trifluoromethyl) phenyl)-4-fluoro-3-nitrobenzamide (**3**) was the potent inhibitor.

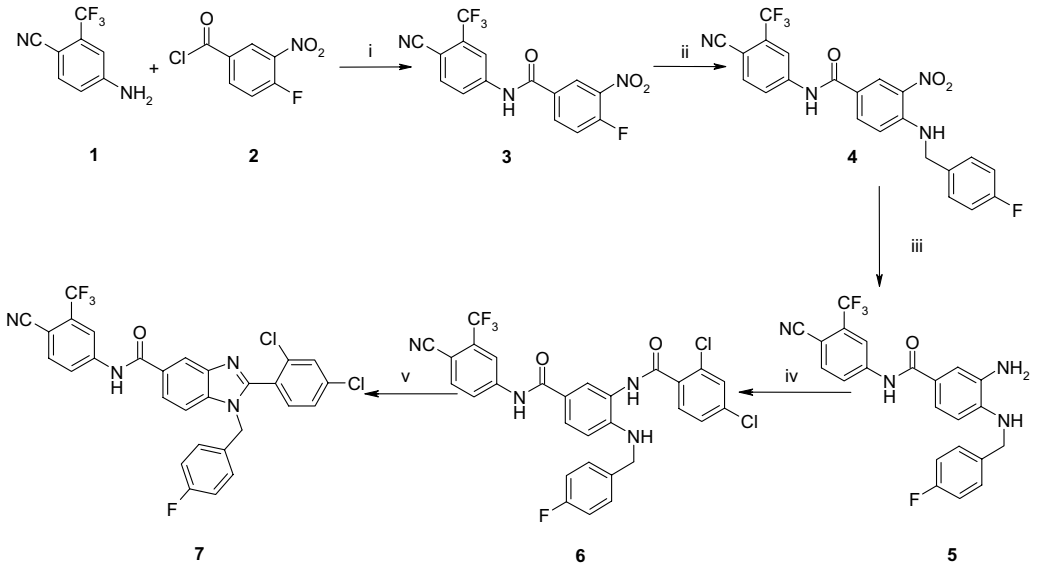
Breast carcinoma is the most common cancer in women worldwide and remains the most frequent cause of malignancy-associated deaths among women. The benzimidazole nucleus is an important pharmacophore in drug discovery.[1] Benzimidazoles are very useful intermediates/subunits for the development of molecules of pharmaceutical or biological interest. Substituted benzimidazole derivatives have diverse therapeutic applications as they exhibit antihistamine,[2,3] antiulcerative,[4] anti-inflammatory, analgesic,[5–9] antioxidant,[10] anti-HIV-1,[11–13] antibacterial,[14–20] antiproliferative,[21,22] antiallergic,[23,24] antitumour,[25–27] antikinase[28,29] and potential anticancer activities.[30–33] The biological relevance of these heteroaromatic groups is due to their being good bioisosteres of biomolecules. Benzimidazoles are also used as biomimetics of guanine residues[34] and benzimidazole derivatives selectively inhibit endothelial cell growth and suppress angiogenesis in vitro and in vivo.[35]

Due to broad spectrum of activities reported so far and in continuation of our research on the synthesis of bioactive heterocycles, we describe here the synthesis of trisubstituted benzimidazole and its precursors and their inhibition of proliferation of MDA-MB-231 human breast cancer cells.

Synthesis of trisubstituted benzimidazole is outlined in Scheme 1. 4-Amino-2-trifluoromethyl-benzonitrile (**1**) was reacted with 4-fluoro-3-nitro-benzoyl chloride (**2**) to produce an amide compound **3**. This reaction required the use of *N,N*-dimethyl acetamide both as base and as solvent. Nucleophilic aromatic substitution of fluorine group with 4-fluoro benzyl amine was carried out using *N,N*-diisopropyl ethylamine as base and *N,N*-dimethyl formamide as solvent at room temperature producing **4**. The aryl nitro group was then reduced to obtain compound **5** by using 5 equiv of aqueous NH_4Cl solution and 20% iron (II) powder using isopropyl alcohol as solvent. The selective acylation of primary aromatic amino moiety in compound **5** was carried out with 2,4-dichloro benzoyl chloride using triethylamine as base and methylene dichloride as solvent producing the compound **6**. Finally,1-(4-fluorobenzyl)-2-(2,4-dichlorophenyl)-*N*-(4-cyano-3-trifluoromethyl)phenyl)-1*H*-benzo[*d*]imidazole-5-carboxamide (**7**) was obtained by cyclisation of compound **6** with glacial acetic acid. All the precursors and substituted benzimidazole compound were obtained in good yield ranging from 81% to 91% after recrystallization with diethyl ether and

Keywords: Trisubstituted benzimidazole; MDA-MB-231; Cell proliferation.
* Corresponding authors. Tel.: +91 821 2412191; fax: +91 821 2412191 (K.S.R.); tel.: +65 65163206; fax: +65 67787643 (G.W.Y.); e-mail addresses: georgeyip@nus.edu.sg; rangappaks@gmail.com; rangappaks@chemistry.uni-mysore.ac.in

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.08.078



Scheme 1. Reagents and conditions: (i) DMA, rt, 4 h; (ii) 4-fluorobenzyl amine, DIPEA, DMF, rt, 3 h; (iii) Fe powder, NH_4Cl, IPA, 70 °C, 6 h; (iv) 2,4-dichlorobenzoyl chloride, TEA, MDC, rt, 3 h; (v) CH_3COOH, 90 °C, 8 h.

the final compound **7** was purified by silica gel column chromatography using ethyl acetate/hexane (4:6) as eluent. All the compounds obtained were characterized by ^{1}H NMR, FTIR, LC/MS and elemental analysis.[36]

We have synthesised the target molecule, substituted benzimidazole **7**, using 4-amino-2-trifluoromethylbenzonitrile (**1**), a starting material, which is used for the synthesis of the well-known antineoplastic drug bicalutamide, and 4-fluoro-3-nitro-benzoyl chloride (**2**) as starting materials to get compound **3**. The latter was confirmed by the presence of –NH peak at 11.19 δ in proton NMR. Similarly, the presence of –N–H stretching frequency at 3352 cm^{-1} and C–O stretching frequency at 1685 cm^{-1} in FTIR spectrum and mass of the compound were confirmed by LC/MS analysis. The intermediate compound **4** was obtained by nucleophilic substitution reaction of compound **3** with 4-fluoro benzylamine. Compound **4** was confirmed by the presence of benzylic proton at 4.70 δ and additional aromatic protons in ^{1}H NMR spectrum and further verified by LC/MS analysis. The compound **5** was obtained by reducing the nitro group in compound **4** and confirmed by the presence of $-NH_2$ peak at 4.88 δ and the absorption peak at 3439 cm^{-1} in FTIR spectra and further confirmed by LC/MS analysis. The compound **6** was obtained by acylation of precursor **5** and this was confirmed by the absence of primary amine peak in FTIR spectrum and presence of additional aromatic proton peak in ^{1}H NMR spectrum. The final target molecule **7** obtained by cyclisation of compound **6** was confirmed by ^{1}H NMR, FTIR, LC/MS and elemental analysis.

Our results revealed that the title compounds showed inhibition of proliferation of MDA-MB-231 human breast cancer cells.[37] Among the compounds tested, the inhibitory activity was found in the order **3** > **7** > **5**. Compound **3** showed maximum inhibition of 60%. Compounds **7** and **5** showed moderate inhibition of 26.1% and 9.8% respectively, and compounds **4** and **6** did not show any inhibition. The significant inhibition by compound **3** may be due to the presence of strong electronegative groups fluorine (F) at para position and nitro ($-NO_2$) at meta position to amide. Replacement of fluorine by electron donating amine decreased the inhibition in compound **4**, reduction of nitro group to amine again increased the inhibition in compound **5**, whereas the selective acylation at meta position reduced the inhibition in compound **6**. The presence of benzimidazole nucleus may be responsible for the moderate inhibition shown by compound **7**.

From the above summary, it can be concluded that the presence of electron withdrawing groups at para position and electron donating groups at meta position to amide may be responsible for the inhibition. But exact interaction of electron withdrawing and donating groups with human breast cancer cells is not known. Further studies are needed to develop still best anti-breast cancer drugs.

In summary, synthesis of trisubstituted benzimidazole and its precursors was carried out with good yield and purity. Compound **3** significantly inhibited MDA-MB-231 human breast cancer cell proliferation, which may be due to presence of electron withdrawing groups at para position and electron donating groups at meta position in amide. Further, synthesis of novel benzimidazole derivatives by changing the substituents at 1st, 2nd and 5th position of the benzimidazole nucleus to enhance the anticancer activity is under progress at our laboratory (Table 1).

Table 1. Percentage inhibition of MDA-MB-231 cell proliferation

Compounds	Inhibition (%)	*P* Value (Student's '*t*'-test)
3	60	0.00
4	0	0.00
5	9.8	0.08
6	0	0.46
7	26.1	0.024

Acknowledgments

The authors are grateful to UGC, Government of India, for financial support under the project UGC-SAP (Phase-I) Programme No. F. 540/10/DRS/2004(SAP-I) and to the National Medical Research Council, Singapore, for financial support from Grants NMRC/0772/2003.DST Project No. DV6/15/DST/2005-06 New Delhi and NMRC/1023/2005 (G.W.Y.) and DST-FIST (order No. SR/FST/CSI-051/2002) grants for the instrument elemental analyser.

References and notes

1. Spasov, A. R.; Iezhitsa, I. N.; Bugaeva, L. I.; Anisimova, V. A. *Khim.-Farm. Zhurn.* **1999**, *33*, 6.
2. Grassmann, S.; Sadek, B.; Ligneau, X.; Elz, S.; Ganellin, C. R.; Arrang, J. M.; Schwartz, J. C.; Stark, H.; Schunack, W. *Eur. J. Pharm. Sci.* **2002**, *15*, 367.
3. Al Muhaimeed, H. *J. Int. Med. Res.* **1997**, *25*, 175.
4. Richter, J. E. *Am. J. Gastroenterol.* **1997**, *92*, 34.
5. Labanauskas, L. K.; Brukstus, A. B.; Gaidelis, P. G.; Buchinskaite, V. A.; Udrenaite, E. B.; Dauksas, V. K. *Pharm. Chem. J.* **2000**, *34*, 353.
6. Sondhi, S. M.; Rajvanshi, S.; Johar, M.; Bharti, N.; Azam, A.; Singh, A. K. *Eur. J. Med. Chem.* **2002**, *37*, 835.
7. Sondhi, S. M.; Singhal, N.; Johar, M.; Reddy, B. S.; Lown, J. W. *Curr. Med. Chem.* **2002**, *9*, 1045.
8. Sondhi, S. M.; Singh, N.; Johar, M.; Kumar, A. . *Bioorg. Med. Chem.* **2005**, *13*, 6158.
9. Sondhi, S. M.; Goyal, R. N.; Lahoti, A. M.; Singh, N.; Shukla, R.; Raghubir, R. *Bioorg. Med. Chem.* **2005**, *13*, 3185.
10. Can-Eke, B.; Puskullu, M. O.; Buyukbingol, E.; Iscan, M. *Chem.-Biol. Interact.* **1998**, *113*, 65.
11. Evans, T. M.; Gardiner, J. M.; Mahmood, N.; Smis, M. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 409.
12. Novelli, F.; Tasso, B.; Sparatore, F.; Sparatore, A. *Chem. Abstr.* **1998**, *128*, 238983j.
13. Bell, C. A.; Dykstra, C. C.; Naiman, N. A.; Cory, M.; Fairley, T. A.; Tidwell, R. R. *Antimicrob. Agents Chemother.* **1993**, *37*, 2668.
14. Klimesova, V.; Koci, J.; Pour, M.; Stachel, J.; Waisser, K.; Kaustova, J. *Eur. J. Med. Chem.* **2002**, *37*, 409.
15. Kumar, B. V.; Vaidya, S. D.; Kumar, R. V.; Bhirud, S. B.; Mane, R. B. *Eur. J. Med. Chem.* **2006**, *41*, 599.
16. Arjmand, F.; Mohani, B.; Ahmad, S. *Eur. J. Med. Chem.* **2005**, *40*, 1103.
17. He, Y.; Yang, J.; Wu, B.; Risen, L.; Swayze, E. E. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1217.
18. He, Y.; Wu, B.; Yang, J.; Robinson, D.; Risen, L.; Ranken, R.; Blyn, L.; Sheng, S.; Swayze, E. E. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3253.
19. Burli, R. W.; Jones, P.; McMinn, D.; Le, Q.; Duan, J. X.; Kaizerman, J. A.; Difuntorum, S.; Moser, H. E. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1259.
20. Burli, R. W.; McMinn, D.; Kaizerman, J. A.; Hu, W.; Ge, Y.; Pack, Q.; Jiang, V.; Gross, M.; Garcia, M.; Tanaka, R.; Moser, H. E. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1253.
21. Garuti, L.; Roberti, M.; Malagoli, M.; Rossi, T.; Castelli, M. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2193.
22. Settimo, A. D.; Settimo, F. D.; Marini, A. M.; Primofiore, G.; Salerno, S.; Viola, G.; Vi, L. D.; Magno, S. M. *Eur. J. Med. Chem.* **1998**, *33*, 685.
23. Beaulieu, C.; Wang, Z.; Denis, D.; Greig, G.; Lamontagne, S.; O'Neill, G.; Slipetz, D.; Wang, J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3195.
24. Nakano, H.; Inoue, T.; Kawasaki, N.; Miyataka, H.; Matsumoto, H.; Taguchi, T.; Inagaki, N.; Nagai, H.; Satoh, T. *Bioorg. Med. Chem.* **2000**, *8*, 373.
25. White, A. W.; Curtin, N. J.; Eastman, B. W.; Golding, B. T.; Hostomsky, Z.; Kyle, S.; Li, J.; Maegley, K. A.; Skalitzky, D. J.; Webber, S. E.; Yu, X.-H.; Griffin, R. J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2433.
26. Lukevics, E.; Arsenyan, P.; Shestakova, I.; Domracheva, I.; Nesterova, A.; Pudova, O. *Eur. J. Med. Chem.* **2001**, *36*, 507.
27. Huang, X.; Suleman, A.; Skibo, E. B. *Bioorg. Chem.* **2000**, *28*, 324.
28. Sondhi, S. M.; Singh, N.; Lahoti, A. M.; Bajaj, K.; Kumar, A.; Lozech, O.; Meijer, L. *Bioorg. Med. Chem.* **2005**, *13*, 4291.
29. Sondhi, S. M.; Singh, N.; Kumar, A.; Lozach, O.; Meijer, L. *Bioorg. Med. Chem.* **2006**, *14*, 3758.
30. Demirayak, S.; Abu Mohsen, U.; Caqri Karaburun, A. *Eur. J. Med. Chem.* **2002**, *37*, 255.
31. Huang, S. T.; Hsei, I. J.; Chen, C. *Bioorg. Med. Chem.* **2006**, *14*, 6106.
32. Kumar, C. C.; Malkowski, M.; Yin, Z.; Tanghetti, E.; Yaremko, B.; Nechuta, T.; Varner, J.; Liu, M.; Smith, E. M.; Neustadt, B.; Presta, M.; Armstrong, L. *Cancer Res.* **2001**, *61*, 2232.
33. White, A. W.; Almassy, R.; Calvert, A. H.; Curtin, N. J.; Griffin, R. J.; Hostomsky, Z.; Maegly, K.; Newell, D. R.; Srivinivasan, S.; Golding, B. T. *J. Med. Chem.* **2000**, *43*, 4084.
34. Boiani, M.; Gonzalez, M. *Mini-Rev. Med. Chem.* **2005**, *5*, 409.
35. Hori, A.; Imaeda, Y.; Kubo, K.; Kusaka, M. *Cancer lett.* **2002**, *183*, 53.
36. *Experimental*: Melting points were determined using SELACO-650 hot stage melting point apparatus and the values are not corrected. Infrared (IR) spectra were recorded using Jasco FTIR-4100 series. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Shimadzu AMX 400-Bruker, 400 MHz spectrometer using DMSO-d_6 and $CDCl_3$ as solvent and TMS as internal standard (chemical shift in δ ppm). Spin multiplets are given as s (singlet), d (doublet), t (triplet) and m (multiplet). Mass and purity were recorded on a LC-MSD-Trap-XCT. Elemental (CHNS) analyses were done on Vario EL III Elementar. Silica gel column chromatography was performed using Merck 7734 silica gel (60–120 mesh) and Merck made TLC plates.
Synthesis of N-(4-cyano-3-(trifluoromethyl)phenyl)-4-fluoro-3-nitrobenzamide (**3**): A solution of 4-amino-2-(trifluoromethyl)benzonitrile (**1**) (10.0 g, 53.7 mmol) in *N*,*N*-dimethyl acetamide (100 mL) was taken and cooled from −5 to 0 °C in an ice bath. 4-Fluoro-3-nitro-benzoyl chloride (**2**) (10.9 g, 53.7 mmol) was added to the cold reaction mixture and stirred for 30 min, and then the reaction mixture was allowed to attain room temperature under stirring for 4 h. The reaction was monitored by TLC. Upon completion, the reaction mixture was quenched with water and filtered under reduced pressure, and the brownish red solid compound obtained (**3**) was recrystallized with diethyl ether. Yield: 90% (17.08 g). Mp 144–146 °C. ^{1}H NMR. (DMSO-d_6, 400 MHz) δ: 11.19 (s, 1H, –CO–NH–), 8.78 (d, 1H, Ar-H), 8.43–8.39 (m, 2H, Ar-H), 8.29–8.26 (d, 1H, Ar-H), 8.18–8.16 (d, 1H, Ar-H), 7.84–7.79 (d, 1H, Ar-H). MS: 354.0. LC purity: 98.43% IR (KBr, cm^{-1}): 3392 (CON–H str), 3059 (–C–H str), 2229 (–CN str), 1685 (–CO str), 1529 and 1326 (–N–O str), 1051 (–C–F str). Anal. Calcd for $C_{15}H_7F_4N_3O_3$ (in %): C, 51.00; H, 2.00; N, 11.90. Found: C, 51.04; H, 2.01; N, 11.86.

Synthesis of 4-(4-fluorobenzylamino)-N-(4-cyano-3-(trifluoromethyl)phenyl)-3-nitrobenzamide (***4***): A solution of *N*-(4-cyano-3-(trifluoromethyl)phenyl)-4-fluoro-3-nitrobenzamide (**3**) (2.0 g, 5.66 mmol) in *N*,*N*-dimethyl formamide was taken. *N*,*N*-diisopropyl ethylamine (2.19 g, 16.98 mmol) was added to the reaction mixture and stirred for 10 min, and then 4-fluorobenzyl amine (0.71 g, 5.66 mmol) was added, and the reaction mixture was stirred for 3 h at room temperature. The reaction was monitored by TLC. Upon completion, the reaction mixture was quenched with water and filtered under reduced pressure and the obtained yellow solid compound (**4**) was recrystallized with diethyl ether. Yield: 91% (2.35 g). Mp 212–214 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.9 (s, 1H, –CO–NH–), 9.1 (t, 1H, –NH–), 8.84 (d, 1H, Ar-H), 8.40 (s, 1H, Ar-H), 8.25 (d, 1H, Ar-H), 8.11 (d, 1H, Ar-H), 8.01 (d, 1H, Ar-H), 7.46–7.42 (m, 2H, Ar-H), 7.2–7.15 (m, 2H, Ar-H), 7.05 (d, 1H, Ar-H), 4.70 (d, 2H, –CH_2–) MS: 459.1. LC purity: 99.47%. IR (KBr, cm^{-1}): 3353 (CON–H str), 3088 (–C–H str), 2224 (–CN str), 1681 (–CO str), 1521 and 1474 (–N–O str), 1049 (–C–F str). Anal. Calcd for $C_{22}H_{14}F_4N_4O_3$ (in %): C, 57.65; H, 3.08; N, 12.22. Found: C, 57.60; H, 3.05; N, 12.25.

*Synthesis of 4-(4-fluorobenzylamino)-3-amino-N-(4-cyano-3-(trifluoromethyl) phenyl) benzamide (**5**)*: A solution of 4-(4-fluorobenzylamino)-*N*-(4-cyano-3-(trifluoromethyl)-phenyl)-3-nitrobenzamide (**4**) (2.0 g) in isopropyl alcohol was taken, 20% (W/W) of iron powder and saturated ammonium chloride solution (5.0 equiv) were added to the reaction mixture and heated to 90 °C for 6 h. The reaction was monitored by TLC. Upon completion, the reaction mixture was filtered through Celite bed, isopropyl alcohol was removed under reduced pressure and residue was extracted with ethyl acetate. Solvent was again removed under reduced pressure to get crude product and recrystallized with diethyl ether to get pure compound **5**. The product obtained was a brown solid. Yield: 87% (1.63 g). Mp 176–178 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.45 (s, 1H, –CO–NH–), 8.23 (d, 1H, Ar-H), 8.06 (d, 1H, Ar-H), 7.41–7.38 (m, 2H, Ar-H), 7.19–7.13 (m, 4H, Ar-H), 6.40–6.38 (d, 1H, Ar-H), 5.99–5.97 (t, 1H, –N–H), 4.88 (s, 2H, NH_2), 4.41–4.39 (d, 2H, –CH_2). MS: 429.1. LC purity: 95.0%. IR (KBr, cm^{-1}): 3439 (–N–H str), 3370 (CON–H str), 3198 (–C–H str), 2228 (–CN str), 1689 (–CO str), 1050 (–C–F str), 750 (–C–Cl). Anal. Calcd for $C_{22}H_{16}F_4N_4O$ (in %): C, 61.68; H, 3.76; N,13.08. Found: C, 61.65; H, 3.72; N, 13.05.

Synthesis of 3-(2,4-dichloro-benzoylamino)-N-(4-cyano-3-trifluoromethyl-phenyl)-4-(4-fluoro-benzylamino)- benzamide (***6***): The compound **5** (1.5 g, 3.5 mmol) in methylene dichloride (15 mL) and triethylamine (1.06 g, 10.50 mmol) were taken and cooled from −5 to 0 °C in an ice bath. 2,4-Dichloro benzoyl chloride (0.73 g, 3.5 mmol) was added to the cold reaction mixture and stirred for 30 min, then the reaction mixture was allowed to attain room temperature with stirring for 3 h. The reaction was monitored by TLC. Upon completion, the reaction mixture was quenched with water and extracted with methylene dichloride. The solvent was removed under reduced pressure, and the obtained white amorphous powder compound (**6**) was recrystallized with diethyl ether. Yield: 86% (1.81 g). Mp 187–189 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.14 (s, 1H, –CO–NH–), 9.01 (s, 1H, –CO–NH–), 8.75 (s, 1H, Ar-H), 8.27 (s, 1H, Ar-H), 8.17–8.14 (d, 1H, Ar-H), 8.03–8.01 (d, 1H, Ar-H), 7.75–7.73 (d, 1H, Ar-H), 7.44–7.39 (t, 1H, Ar-H), 7.27–7.23 (d, 1H, Ar-H), 6.98–6.95 (d, 2H, Ar-H). 6.93–6.91 (t, 4H, Ar-H), 5.22 (s, 2H, –CH_2), 5.11 (t, 1H, –NH) MS: 601. LC purity: 97.12%. IR (KBr, cm^{-1}): 3430 (N–H str), 3379 (CON–H str), 3128 (–C–H str), 2226 (–CN str), 1681 (–CO str), 1048 (–C–F str), 747 (–C–Cl). Anal. Calcd for $C_{29}H_{18}Cl_2F_4N_4O_2$ (in %): C, 57.92, H, 3.02, N, 9.32. Found: C,57.85; H, 3.07; N, 9.29.

Synthesis of 1-(4-fluorobenzyl)-2-(2,4-dichlorophenyl)-N-(4-cyano-3-(trifluoromethyl)phenyl)-1H-benzo[d]imidazole-5-carboxamide (***7***): Obtained compound **6** (1.5 g, 2.5 mmol) was taken in glacial acetic acid and allowed the reaction to proceed at 90 °C for 8 h. The reaction was monitored by TLC. Upon completion, the reaction mixture was quenched with water and filtered under reduced pressure, and the obtained white amorphous powder compound **7** was purified by silica gel column chromatography using ethyl acetate and hexane as eluent (4:6). Yield: 81% (1.18 g). Mp 222–224 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.14 (s, 1H, –CO–NH–), 8.75 (s, 1H, Ar-H), 8.27 (s, 1H, Ar-H), 8.17–8.14 (d, 1H, Ar-H), 8.03–8.01 (d, 1H, Ar-H), 7.75–7.73 (d, 1H, Ar-H), 7.44–7.39 (d, 1H, Ar-H), 7.27–7.23 (d, 1H, Ar-H), 6.98–6.95 (d, 2H, Ar-H), 6.93–6.91 (m, 4H, Ar-H), 5.22 (s, 2H, –CH_2) MS: 583.0. LC purity: 99.12%. IR (KBr, cm^{-1}): 3379 (CON–H str), 3128 (–C–H str), 2226 (–CN str), 1681 (–CO str), 1048 (–C–F str), 747 (–C–Cl). Anal. Calcd for $C_{29}H_{16}Cl_2F_4N_4O$ (in %): C, 59.71; H, 2.76; N, 9.60. Found: C, 59.65; H, 2.70; N, 9.55.

37. *Biology: Culturing human breast cancer cell line*
MDA-MB-231 human breast cancer cells were grown in RPMI medium (Sigma), supplemented with 10% foetal bovine serum (FBS). The cells were maintained at 37 °C in a 5% CO_2 incubator. They were subsequently dislodged from the substratum by 1× trypsin treatment for 5 min followed by inactivation using FBS.
Cell proliferation assay: The MTS assay was performed using the Promega CellTiter 96® aqueous non-radioactive cell proliferation assay kit as previously described.[38] Briefly, MDA-MB-231 cells were seeded onto the wells of 96-well plates at a density of 2000 cells/well. The test compounds at 2 mM final concentration in 1% DMSO were added and the cultures continued for 72 h. Replenishment with fresh compounds was done after the first 48 h. At the end of 72 h of treatment, the cultured medium was removed and 20 μl/well of combined MTS/PMS solution was added and incubated for 4 h. The absorbance at 490 nm was recorded using an ELISA plate reader.
38. Zou, X. H.; Foong, W. C.; Cao, T.; Bay, B. H.; Ouyang, H. W.; Yip, G. W. *J. Dent. Res.* **2004**, *83*, 880.