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ORIGINAL ARTICLE

Synthesis and biological evaluation of 2-(phenyl)-3H-benzo[*d*]imidazole-5-carboxylic acids and its methyl esters as potent anti-breast cancer agents

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Abstract A series of novel substituted 2-(phenyl)-3*H*-benzo[*d*]imidazole-5-carboxylic acids (**1a–1j**) and its methyl esters (**2a–2f**) were synthesized and examined for their antiproliferative effects against three breast cancer cell lines (MDA-MB231, MDA-MB468 and MCF7) *in vitro*. Most of the compounds exhibited comparable or greater antiproliferative effects than the reference compound cisplatin. Compound **2e** bearing 5-fluoro-2-hydroxyphenyl substituent was found to be the most active derivative of the series with GI_{50} values of **6.23, 4.09** and **0.18** μM against MDA-MB468, MDA-MB231 and MCF7 breast cancer cell lines, respectively. Our findings described here exemplify the usefulness of the title compounds as a lead for the development of more effective cancer therapeutics for the treatment of breast cancer.

1. Introduction

Cancer is still one of the leading causes of death worldwide. The death rate from various types of cancer continues to rise worldwide with an estimated 12 million deaths in 2030 (Solomon et al., 2009; Patel et al., 2011). One in ten of all new types of cancer diagnosed around the world each year is breast cancer and is the most common cancer in women in both developing and developed countries (www.who.int/cancer/detection/breastcancer/

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en). Estrogen receptor (ER)-negative breast cancer accounts for approximately 30% of all breast cancer diagnosed (Somers-Edgar et al., 2011; Yadav et al., 2011; Farag et al., 2010). Chemotherapy has been the most frequently used treatment for breast cancer and other cancers till date. However, some normal proliferating cells are destroyed as well by this method of treatment (Wang et al., 2009). Hence, there is an intense worldwide search for identifying new drugs that are more effective and safe for the prevention and treatment of cancer.

A survey of the literature in this area shows that many heterocyclic scaffolds have been investigated as anti-breast cancer agents (Al-Said et al., 2011). Benzimidazoles are a useful structural motif for the development of molecules of pharmaceutical or biological interests (Taher et al., 2011). Benzimidazole derivatives are structural isosteres of naturally occurring nucleotides, which allow them to interact easily with the biomolecules in living organisms. Benzimidazole derivatives possess various biological activities and the optimization of benzimidazole-based structures has resulted in various drugs that are currently in the market, including omeprazole (proton pump inhibitor), pimobendan (ionodilator), and mebendazole (anthelmintic) (Starcevic et al., 2007; Alamgir et al., 2007; Weber et al., 2007; Spasov et al., 1999). Some recent reports have focused on the profound anticancer activities shown by benzimidazoles and its derivatives, resulting in renewed interest in this class of molecules. Hoechst 33258 (National Cancer Institute (NCI) entry number 322921) is a head-to-tail bis-benzimidazole compound that specifically recognizes three consecutive A:T base pairs in the minor groove of B form DNA (Seaton et al., 2003). This compound has been serving as a model compound for biochemical and biophysical studies of drugs that directly bind to the DNA minor groove, causing cytotoxicity. 2-Substituted benzimidazoles (bis-benzimidazole derivatives) were also found to be cytotoxic against breast adenocarcinoma (MCF7) and skin epidermoid carcinoma (A431) by interfering DNA topoisomerase I activity DNA (Seaton et al., 2003; Bielawski et al., 2004; Refaat, 2010). Heterocyclic benzimidazole derivatives bearing amidino substituents at the C-5 position of a benzimidazole ring and other heterocyclic nuclei at the C-2 position showed selectivity toward the MCF7 breast cancer cells (Starcevic et al., 2007).

These broad antitumor properties exhibited by benzimidazoles and our continuing interest in the exploration of novel heterocyclic scaffolds for anticancer activity (Patel et al., 2011; Moorthy et al., 2009, 2010) have prompted us to synthesize a series of novel benzo[*d*]imidazole-5-carboxylic acid derivatives and to evaluate these compounds against three breast cancer cell lines.

2. Materials and methods

All commercially available chemicals and solvents used were of reagent grade and used without further purification. ^{1}H NMR spectra were obtained with a Bruker Avance II 400 NMR spectrometer. Chemical shifts were recorded in parts per million (ppm) and were reported relative to the TMS. Mass spectral data were measured on an Applied Biosystem Qtrap 3200 LC-MS/MS system in ESI mode. The IR spectra of the synthesized compounds were recorded on Schimadzu FT-IR 8400S. Elemental analysis was performed on a Vario Micro cube CHNS analyzer of Elementar Make. Melting points of the compounds were determined using a Veego digital melting point apparatus and are reported uncorrected. Reactions were monitored by TLC using pre-coated silica gel aluminum plates (Kieselgel 60, 254, E. Merck, Germany); zones were detected visually under ultraviolet irradiation.

2.1. General procedure for the synthesis of *1a–1j* and *2a–2f*

To a solution of aldehyde (2.0 mmol) in 6.5 mL of *N*,*N*-dimethyl acetamide were added 3,4 diamino benzoic acid or its methyl ester (2.0 mmol) and $Na_2S_2O_5$ (2.4 mmol). The mixture was heated to 100 °C for 6.5–12 h until TLC confirms the completion of the reaction. The reaction mixture was then cooled, diluted with ethyl acetate (5×25 mL), dried ($MgSO_4$), and concentrated *in vacuo*. The solid obtained was collected on a sintered-glass filter and washed with dichloromethane (3x) to provide the desired compound.

2.1.1. 2-(2-Hydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (*1a*)

67% yield; M.P > 300 °C; IR (KBr) ν (cm^{-1}): 3319 (–NH), 3063 (Ar–C–H), 1683 (–C═O), 1631 (ArC–C), 1261 (–C–N); ^{1}H NMR (400 MHz, DMSO-d6): δ (ppm) 12.93 (br, s, 1H, NH), 8.24 (s, 1H, Ar H), 8.09 (d, 1H, *J* = 7.6 Hz, Ar H), 7.90 (d, 1H, *J* = 8.4 Hz, Ar H), 7.73 (d, 1H, *J* = 8.0 Hz, Ar H), 7.42 (t, 1H, *J* = 7.6 Hz, Ar H), 7.03-7.08 (m, 2H, Ar H); LC–MS analysis (M + H): 255.2 (calculated 254.24); **Elemental Analysis: Calcd. (Found) (%) for $C_{14}H_{10}N_2O_3$**: C 66.14 (66.18), H 3.96 (3.96), N 11.02 (11.00).

2.1.2. 2-(2,3-Dihydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (*1b*)

51% yield; M.P. 285 °C; IR(KBr) ν (cm^{-1}): 3284 (–NH), 2989 (Ar–C–H), 1685 (–C═O), 1622 (ArC–C), 1271 (–C–N); ^{1}H NMR (400 MHz, DMSO-d6): δ (ppm) 13.38 (br, s, 1H, COOH) 12.81 (br, s, 1H, NH), 9.26 (s, 1H, Ar OH), 8.22 (s, 1H, Ar H), 7.9 (d, 1H, *J* = 7.6 Hz, Ar H), 7.72 (s, 1H), 7.52 (d, 1H, *J* = 8 Hz, Ar H), 6.95 (d, 1H, *J* = 7.6 Hz, Ar H), 6.86 (t, 1H, *J* = 8 Hz, Ar H); LC–MS analysis (M + H): 271.0 (calculated 270.24); **Elemental Analysis: Calcd. (Found) (%) for $C_{14}H_{10}N_2O_4$**: C 62.22 (62.27), H 3.73 (3.71), N 10.37 (10.36).

2.1.3. 2-(2,4-Dihydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (*1c*)

69% yield; M.P. 253 °C; IR(KBr) ν (cm^{-1}): 3242 (–NH), 3076 (Ar–C–H), 1708 (–C═O), 1614 (Ar C–C), 1267 (–C–N); ^{1}H NMR (400 MHz, DMSO-d6): δ (ppm) 12.78 (br, s, 1H, NH), 9.66 (s, 1H, OH), 8.19 (s, 1H, Ar H), 7. 75–7.88 (m, 4H, Ar H), 7.55 (s, 1H, Ar H); LC–MS analysis (M + H): 271.2 (calculated 270.24); **Elemental Analysis: Calcd. (Found) (%) for $C_{14}H_{10}N_2O_4$**: C 62.22 (62.28), H 3.73 (3.70), N 19.27 (19.28).

2.1.4. 2-(2-Hydroxy-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (*1d*)

56% yield; M.P. 273 °C; IR (KBr) ν (cm^{-1}): 3524.06 (–NH), 3068.85 (Ar–C–H), 1683.91 (–C═O), 1624.12 (Ar–C–C), 1257.63 (–C–N); ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 13.16 (br, s, 1H, NH) 8.22 (s, 1H, Ar H) 7.88(d, 1H,

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J = 8.28 Hz, Ar H), 7.58(dd, 2H, *J* = 7.96, 1.28 Hz, Ar H), 6.98 (dd,1H, *J* = 8.0, 1.2 Hz, Ar H), 6.88 (t, 1H, *J* = 7.9 Hz, Ar H), 3.86 (s, 3H, OCH_3); LC–MS analysis (M + H): 285.2 (calculated 284.27); **Elemental Analysis: Calcd. (Found) (%) for $C_{15}H_{12}N_2O_4$**: C 63.38 (63.43), H 4.25 (4.26), N 9.85 (9.82).

*2.1.5. 2-(5-Fluoro-2-hydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (**1e**)*

72% yield; M.P. 296 °C; IR (KBr) ν (cm^{-1}): 3321 (–NH), 3080 (Ar–C–H), 1683 (–C═O), 1259 (–C–N), 1506 (ArC–C); ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 13.28 (br, s, 1H, COOH) 12.71 (br s, 1H, NH), 8.22 (s, 1H, Ar H), 7.83–7.91 (m, 2H, Ar H), 7.63 (s, 1H, Ar H), 6.96–7.13 (m, 2H, Ar H); LC–MS analysis (M–H): 270.8 (calculated 272.06); **Elemental Analysis: Calcd. (Found) (%) for $C_{14}H_9FN_2O_3$**: C 61.77 (61.76), H 3.33 (3.30), N 10.29 (10.27).

*2.1.6. 2-(5-Bromo-2-hydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (**1f**)*

68% yield; M.P. 282 °C; IR (KBr) ν (cm^{-1}): 3238 (–NH), 3068 (Ar–C–H), 1693 (–C═O), 1622 (Ar C–C), 1257 (–C–N), 1058.42 (–Br); ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 13.86 (br s, 1H, COOH), 12.93 (br s, 1H, NH), 7.78–8.33 (m, 6H, Ar H); LC–MS analysis (M + H): 335 (calculated 333.14); **Elemental Analysis: Calcd. (Found) (%) for $C_{14}H_9BrN_2O_3$**: C 50.47 (50.50), H 2.72 (2.74), N 8.41 (8.38).

*2.1.7. 2-(2-Hydroxy-5-nitrophenyl)-1H-benzo[d]imidazole-5-carboxylic acid (**1g**)*

58% yield; M.P. 274 °C; IR(KBr) ν (cm^{-1}): 3245 (–NH), 1705 (–C═O), 1614 (Ar C–C), 1535.39 (NO2), 1288 (–C–N); ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 13.76 (br, s, 1H, NH), 9.09 (s, 1H, Ar H) 8.25 (s, 1H, Aromatic CH) 8.16 (dd, 1H, Aromatic CH J = 2.64 Hz, 9.16 Hz), 7.92 (dd, 1H, *J* = 1.28 Hz, 8.48 Hz, Ar H), 7.6 (d, 1H, *J* = 8.4 Hz, Ar H), 7.1 (d, 1H, *J* = 9.12 Hz, Ar H); LC–MS analysis (M + H): 300.2 (calculated 299.24); **Elemental Analysis: Calcd. (Found) (%) for $C_{14}H_9N_3O_5$**: C 56.19 (56.18), H 3.03 (3.04), N 14.04 (14.06).

*2.1.8. 2-(3,4-Dihydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (**1h**)*

62% yield; M.P. 284 °C; IR (KBr) ν (cm^{-1}): 3342 (–NH), 3068 (Ar–C–H), 1627 (ArC–C), 1294 (–C–N); ^1H NMR (400 MHz, DMSO-d6): δ (ppm) 12.68 (br s, 1H, NH), 9.26 (br s, 1H, OH), 8.99 (br s, 1H, OH), 8.12 (s, 1H, Ar H), 7.77 (dd, 1H, *J* = 9.76 Hz, 1.36 Hz, Ar H), 7.61 (s, 1H, Ar H), 7.47 (dd, 2H, *J* = 2.04, 8.24 Hz, Ar H), 6.85 (d, 1H, *J* = 8.32 Hz, Ar H); LC–MS analysis (M + H): 271 (calculated 270.24); **Elemental Analysis: Calcd. (Found) (%) for $C_{14}H_{10}N_2O_4$**: C 62.22 (62.25), H 3.73 (3.71), N 10.37 (10.38).

*2.1.9. 2-(4-Hydroxy-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (**1i**)*

55% yield; M.P. 257 °C; IR (KBr) ν (cm^{-1}): 3317 (–NH), 3088 (Ar–C–H), 1672 (–C═O), 1294 (–C–N); ^1H NMR (400 MHz, DMSO-d6): δ (ppm) 12.93 (br, s, 1H, NH), 9.41 (s, 1H, OH), 8.14 (s, 1H, Ar H), 7.8 (dd, 1H, *J* = 8.4 Hz, 1.32 Hz, Ar H), 7.74 (s, 1H, Ar H), 7.61 (d, 1H, *J* = 8.2 Hz, 1.8 Hz, Ar H), 7.52 (d, 1H, *J* = 8.3 Hz, Ar H), 6.90 (d, 1H, J = 8.2 Hz, Ar H), 3.91 (s, 3H, OCH_3); LC–MS analysis (M + H): 285.2 (calculated 284.27); **Elemental Analysis: Calcd. (Found) (%) for $C_{15}H_{12}N_2O_4$**: C 63.38 (63.38), H 4.25 (4.26), N 9.85 (9.86).

*2.1.10. 2-(3,4,5-Trihydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (**1j**)*

54% yield; M.P. 267 °C; IR (KBr) ν (cm^{-1}): 3321 (–NH), 3080 (Ar–C–H), 1683 (–C═O), 1625 (Ar C–C), 1259 (–C–N); ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 12.58 (br s, 1H, NH), 8.86 (br s, 2H, OH), 8.42 (br, s, 1H, OH), 8.12 (s, 1H, Ar H), 7.77 (dd, 1H, *J* = 8.4 Hz, 1.36 Hz, Ar H), 7.43 (d, 1H, *J* = 8.2, Ar H), 7.17 (s, 2H, Ar H); LC–MS analysis (M + H): 287 (calculated 286.24); **Elemental Analysis: Calcd. (Found) (%) for $C_{14}H_{10}N_2O_5$**: C 58.74 (58.78), H 3.52 (3.50), N 9.79 (9.78).

*2.1.11. Methyl 2-(2-hydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (**2a**)*

67% yield; M.P. 216 °C; IR (KBr) ν (cm^{-1}): 3311 (–NH), 3064 (Ar–C–H), 1720 (–C═O), 1253 (–C–N), 1213 (–CO–O), 1593 (ArC–C); ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 13.0 (br s, 1H, NH), 8.23 (s, 1H, Ar H), 7.96 (d, 1H, *J* = 7.8 Hz, Ar H), 7.88(dd, 1H, *J* = 8.46 Hz, 1.4 Hz, Ar H), 7.58 (d, 1H, *J* = 8.6 Hz, Ar H), 7.31 (t, 1H, *J* = 7.72 Hz, Ar H), 6.97 (d, 1H, *J* = 8.28 Hz, Ar H), 6.92 (t, 1H, *J* = 7.72 Hz, Ar H), 3.86 (s, 3H, CH_3); LC–MS analysis (M + H): 270.8 (calculated 272.06); **Elemental Analysis: Calcd. (Found) (%) for $C_{15}H_{12}N_2O_3$**: C 67.16 (67.10), H 4.51 (4.55), N 10.44 (10.46).

*2.1.12. Methyl 2-(2,3-dihydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (**2b**)*

59% yield; M.P. 241 °C; IR (KBr) ν (cm^{-1}): 3298 (–NH), 3078 (Ar–C–H), 1687 (–C═O), 1271 (–C–N), 1242 (–CO–O), 1539 (ArC–C); ^1H NMR (400 MHz, DMSO-d6): δ (ppm) 13.12 (br, s, 1H, NH), 8.86 (br, s, 1H, Ar OH), 8.22 (s, 1H, Ar H), 7.88 (dd, 1H, *J* = 8.42, 1.32 Hz, Ar H), 7.45(dd, 1H, *J* = 7.94, 1.32 Hz, Ar H), 6.9 (dd, 1H, *J* = 7.86,1.32 Ar H), 6.76 (t, 1H, *J* = 7.88 Hz, Ar H), 3.86 (s, 3H, CH_3); LC–MS analysis (M –H): 282.8 (calculated 284.08); **Elemental Analysis: Calcd. (Found) (%) for $C_{15}H_{12}N_2O_4$**: C 63.38 (63.35), H 4.25 (4.27), N 9.85 (9.87).

*2.1.13. Methyl 2-(2-hydroxy-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (**2c**)*

74% yield; M.P. 234 °C (degrades); IR (KBr) ν (cm^{-1}): 3311 (–NH), 3064 (Ar–C–H), 1720 (–C═O), 1253 (–C–N), 1593 (ArC–C); ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 13.21 (br, s, 1H, NH), 8.2 (s, 1H, Ar H), 7.86 (d, 1H, *J* = 8.48 Hz, Ar H), 7.56–7.61 (m, 2H, Ar H), 6.98 (d, 1H, *J* = 7.6 Hz, Ar H), 6.87 (t, 1H, *J* = 8.0, Ar H), 3.85 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3); LC–MS analysis (M + H): 299.0 (calculated 298.1); **Elemental Analysis: Calcd. (Found) (%) for $C_{16}H_{14}N_2O_4$**: C 64.42 (64.40), H 4.73 (4.75), N 9.39 (9.38).

*2.1.14. Methyl 2-(2-hydroxy-5-nitrophenyl)-1H-benzo[d]imidazole-5-carboxylate (**2d**)*

63% yield; M.P. 223 °C; IR (KBr) ν (cm^{-1}): 3302 (–NH), 3008 (Ar–C–H), 1708 (–C═O), 1278 (–C–N), 1220 (–CO–O), 1591 (Ar C–C); ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 13.16 (br s, 1H, NH), 8.41 (s, 1H, Ar H), 8.23 (s, 1H, Ar H), 7.87

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– 7.94 (m, 2H, Ar H), 7.78 (d, 1H, J = 8.2 Hz, Ar H), 7.2 (d, 1H, J = 7.6 Hz, Ar H), 3.85 (s, 3H, CH_3); LC–MS analysis (M + H): 314.0 (calculated 313.07); **Elemental Analysis: Calcd. (Found) (%) for $C_{15}H_{11}N_3O_5$**: C 57.51 (57.55), H 3.54 (3.52), N 13.41 (13.40).

2.1.15. Methyl 2-(5-fluoro-2-hydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (**2e**)

72% yield; M.P. 239 °C; IR (KBr) ν (cm^{-1}): 3333 (–NH), 3057 (Ar–C–H), 1722 (–C═O), 1255 (–C–N), 1213 (–CO–O) 1535 (ArC–C); ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 13.12 (br s, 1H, NH), 8.22 (s, 1H, Ar H), 7.82–7.88 (m, 2H, Ar H), 7.67(s, 1H, Ar H), 6.96–7.12 (m, 2H, Ar H), 3.85 (s, 3H, CH_3); LC–MS analysis (M + H): 285.0 (calculated 286.08); **Elemental Analysis: Calcd. (Found) (%) for $C_{15}H_{11}FN_2O_3$**: C 62.94 (62.90), H 3.87 (3.89), N 9.79 (9.80).

2.1.16. Methyl 2-(3,4-dihydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (**2f**)

54% yield; M.P 228 °C; IR (KBr) ν (cm^{-1}): 3363 (–NH), 3282 (–OH), 1701 (–C═O), 1259 (–C–N), 1211 (–CO–O), 1600 (ArC–C); ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 13.16 (br s, 1H, NH), 10.21 (br s, 1H, Ar OH), 9.57 (br, s, 1H, Ar OH), 8.29 (s, 1H, Ar H), 8.06 (dd, 1H, J = 8.52,1.4 Hz, Ar H), 7.68 – 7.77 (m, 3H, Ar H), 7.02 (d, 1H, J = 8.4), 3.87 (s, 3H, CH_3); LC–MS analysis (M + H): 283.0 (calculated 284.08); **Elemental Analysis: Calcd. (Found) (%) for $C_{15}H_{12}N_2O_4$**: C 63.38 (63.35), H 4.25 (4.27), N 9.85 (9.86).

2.2. Anticancer activity evaluation

2.2.1. Cell lines

The human MDA-MB468, MDA-MB231 and MCF-7 breast cancer cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan UT) and 2 mM L-glutamine. Cells were grown at 37 °C with 5% CO_2, 95% air under the humidified conditions. The stock solution was diluted in culture medium (0.1–100 μM) immediately before use. The final concentration of DMSO in the SRB-based cytotoxicity assays did not exceed 0.1%. To rule out that the DMSO concentration used may affect cell cytotoxicity, culture medium containing equivalent concentration of DMSO was used as a negative control in all experiments. In all studies, the concentration of DMSO used did not notably show any cytotoxicity.

2.2.2. Reagents

Cisplatin was purchased from Sigma–Aldrich Canada Ltd (Oakaville, ON, Canada). All the compounds were dissolved in 10–20 mM dimethyl sulfoxide (DMSO) and stored at −20 °C until use. The stock solution was diluted in culture medium (0.1–100 μM) immediately before use. The final concentration of DMSO in the SRB-based cytotoxicity assays did not exceed 0.1%. To rule out that the DMSO concentration used may affect cell cytotoxicity, culture medium containing equivalent concentration of DMSO was used as a negative control in all experiments. In all studies, the concentration of DMSO used did not notably show any cytotoxicity.

2.2.3. SRB assay

Cytotoxic effects were determined by a Sulforhodamine B (SRB)-based protocol (Solomon et al., 2009; Vichai and Kirtikara, 2006). For a typical screening experiment, 5,000–10,000 cells were inoculated into 100 μL medium per well of a 96-well microtiter plate as described previously (Solomon et al., 2009; Skehan et al., 1990; Vichai and Kirtikara, 2006). Briefly, after the inoculation, the microtiter plate was incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h, prior to the addition of experimental drugs. Some of the sample wells were fixed with 25 μL of 50% trichloroacetic acid (TCA) as a control of the cell population for each cell line at the time of drug addition (Tz). An aliquot of the frozen stock was thawed and diluted to the desired final maximum test-concentration with complete medium. Two- to ten-fold serial dilutions were made to provide a total of seven drug concentrations (and a control [C]). Following the addition of drugs, the culture plate was incubated for additional 48 h. Cells were fixed *in situ* by slowly adding 25 μL of cold 50% (w/v) TCA (final concentration, 10% TCA), and were then incubated for 60 min at 4 °C. The supernatant was discarded, and the plate was washed five times with tap water, followed by air-drying. 50 μL of SRB solution at 0.4% (w/v) in 1% acetic acid was added to each well, and the plate was incubated for > 30 min at room temperature. Unbound SRB was removed by five washes with tap water, followed by air-drying. The cells "stained" with SRB were solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515–564 nm. The relative growth rate (%) was calculated for each of the compound concentrations according to the following formula:

$$(\mathrm{Ti} - \mathrm{Tz})/(\mathrm{C} - \mathrm{Tz}) \times 100 \quad (1)$$

In the formula, Tz denotes time zero, C denotes control growth and Ti denotes the OD for different concentrations of tested compounds. The GI_{50} for each compound was obtained from a non-linear Sigmoidal dose–response (variable slope) curve which is fitted by GraphPad Prism v.4.03 software. Values were calculated for each of these parameters if the level of activity was reached. However, if the effect was not reached or was exceeded, the value for that parameter was expressed as greater or less than the maximum or minimum concentration tested.

3. Results and discussion

The synthesis of (2-(hydroxyphenyl)-3*H*-benzo[*d*]imidazole-5-carboxylic acids was accomplished with the method reported by Scheme 1 (Arienti et al., 2005). The compounds **1a–1j** were obtained by condensing equimolar quantities of commercially available hydroxy benzaldehydes with 3-, 4-diamino benzoic acid in dimethyl acetamide in the presence of sodium metabisulfite ($Na_2S_2O_5$) at 100 °C. The structures of synthesized compounds were determined using different spectroscopic techniques, including ^{1}H NMR, IR, and LC–MS analyses and elemental analysis.

The synthesized (2-(hydroxyphenyl)-3*H*-benzo[*d*]imidazole-5-carboxylic acids and its methyl esters were evaluated for their antiproliferative activity against a panel of three breast cancer cell lines: MDA-MB468 (a PTEN defective, intermediately differentiated, EGFR positive breast adenocarcinoma

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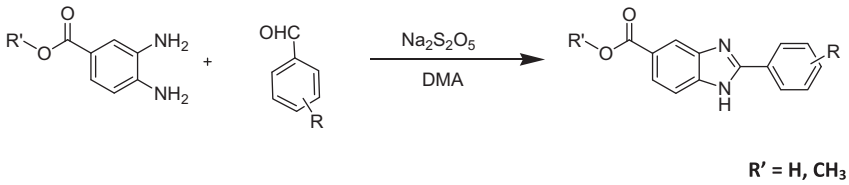
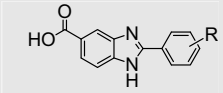
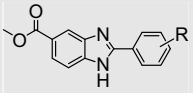


Scheme 1 Synthesis of 2-(phenyl)-3*H*-benzo[*d*]imidazole-5-carboxylic acids and its methyl ester derivatives.

Table 1 Structure and anti-breast cancer activity of 2-(phenyl)-3*H*-benzo[*d*]imidazole-5-carboxylic acids (**1a–1j**) and its methyl ester derivatives (**2a-2f**).



(**1a to 1j**)



(**2a-2f**)

Code	GI_{50} (μM)[a,b]			
		MDA-MB231	MDA-MB468	MCF7
1a	2-OH	42.21	122.05	48.48
1b	2,3-di OH	65.53	91.91	70.45
1c	2,4-di OH	75.61	101.35	98.48
1d	2-OH, 3-OMe	70.73	83.26	26.48
1e	2- OH, 5-F	33.25	29.14	25.47
1f	2-OH, 5-Br	85.21	75.65	73.69
1g	2-OH,5-NO2	56.23	48.57	34.21
1h	3,4-di OH	12.85	11.85	9.23
1i	3 MeO, 4-OH	25.87	21.98	18.68
1j	3,4,5-tri OH	46.32	98.25	75.36
2a	2-OH	95.26	85.08	74.12
2b	2,3-di OH	11.25	4.01	3.70
2c	2-OH, 3-OMe	12.35	11.52	9.52
2d	2-OH,5-NO2	3.57	4.03	7.79
2e	2- OH, 5-F	6.23	4.09	0.18
2f	3,4-di OH	34.20	26.46	19.84
Ref.	Cisplatin	23.65	31.02	25.77

[a] GI_{50} values were calculated from Sigmoidal dose response curves (variable slope), which were generated with GraphPad Prism V. 4.02 (GraphPad Software Inc.).
[b] Values are the mean of triplicates of at least two independent experiments.

cell line), MDA-MB231 (undifferentiated estrogen receptor-negative basal-like breast cancer) and MCF7 (well differentiated, p53+/−, invasive ductal breast carcinoma). Each compound stored at 20 mM was diluted from 100 μM to 0.0064 μM by fivefold serial dilutions. Cells were treated with each compound for 48 h, followed by measuring cell growth rates by SRB-based spectrophotometry. The reading of SRB staining value is known to accurately reflect the levels of total cellular macromolecules/cell growth/proliferation (Skehan et al., 1990). The GI_{50} concentration for each compound was calculated with reference to a control sample, which represents the concentration that results in a 50% decrease in cell growth/proliferation after 48 h incubation in the presence of the drug. For each compound, GI_{50} was deduced from Sigmoidal dose–response curves that were generated with data obtained from two independent experiments carried out each in triplicate and presented in Table 1. The data of cisplatin was included as reference standard. The resultant data showed that, among 16 compounds studied, eight compounds exhibited substantial antiproliferative effects on all three breast cancer cell lines examined. The results also indicated that the methyl esters (**2**) showed greater growth inhibitory potency than free carboxylic acid derivatives (**1**).

Among benzo[*d*]imidazole-5-carboxylic acids, compounds with the 2-hydroxyl group in the aryl ring (**1a–g**) were the least potent with an exception of compound **1e** which showed an inhibitory profile comparable to cisplatin. Compound **1a** with a hydroxyl group at the ortho position of the aryl ring showed

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GI_{50} values of ~50 μM against MDA-MB231 and MCF7 and 122 μM against MDA-MB468. Introduction of an additional hydroxyl group in compound **1a** either at meta (**1b**) or para position (**1c**) of the aryl ring resulted in decreased antiproliferative effects on all the three cancer cells. Substitution of the 3-methoxy group in compound **1a** slightly improved the growth inhibitory potency against MDA-MB468 and MCF7 cells. Fluorine substitution at the fifth position of the aryl rings (**1e**) resulted in a marked increase in the anti proliferative activity against the three breast cancer cells. However, replacement of fluorine by a larger halogen atom, bromine (**1f**) led to marked reduction of antiproliferative effect on MDA-MB231 and MCF7 cells. Introduction of the electron withdrawing nitro group at fifth position of the aryl ring (compound **1g**) resulted in modest improvement in the growth inhibitory activity against MDA-MB468 and MCF7 cells.

Compound **1h** with 3, 4-dihydroxyl substitution in the aryl ring was found to be the most potent with GI_{50} values of 12.85, 11.85 and 9.23 μM against MDA-MB468, MDA-MB231 and MCF breast cancer cells respectively. Introduction of an additional hydroxyl group in the aryl ring to give a trihydroxyl derivative **1j** led to a drastic reduction in antiproliferative activity. Compound **1i** which is a mono methylated derivative of compound **1h** showed a one fold decrease in antiproliferative potency against the three breast cancer cells.

Benzo[*d*]imidazole-5-carboxylic acids showed only modest potency against the studied breast cancer cell lines contrary to our expectations. The poor anti-breast cancer profile exhibited these compounds which may be due to the ionization of free carboxylic acid moiety to a charged carboxylate anion in aqueous conditions hence impeding the cellular permeability of these studied compounds. A similar phenomenon was recorded by Gowda et al. (2009) and they had overcome this problem by transformation of the free carboxylic group to an ester. Related to the foregoing, methyl ester analogs of the benzo[*d*]imidazole-5-carboxylic acid were synthesized and subsequently evaluated for their antiproliferative effects on the breast cancer cells. The esterification of the carboxylic group in the title compounds led to mixed results. The esterification caused a dramatic increase in the cytotoxicity to the majority of compounds (**2b–2e**) while decreasing potency to a few compounds (**2a** and **2f**). Compound **2e**, the methyl ester derivative of 2-(5-fluoro-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid **(1e)** displayed excellent growth inhibitory activity against estrogen receptor dependent MCF7 cell line in submicromolar concentration (GI_{50} = 0.18 μM) which is greater than a hundred fold improvement than its unesterified counterpart (**1e**). Furthermore, esterification also improved the antiproliferative activity of compound **1e** against MDA-MB231 and MDA-MB468 cells by greater than fivefold. Compound **2d** with 2-hydroxy, 5-nitro substitutions in the phenyl ring showed greater potency (GI_{50} < 5 μM) against the ER negative MDA-MB231 and MDA-MB468 cells than the ER-positive MCF7. Another ester derivative with comparable antiproliferative activity profile is compound **2b** with 2-, 3-dihydroxy substitution in the phenyl ring. Compound **2b** shows greater potency than reference standard cisplatin, as its GI_{50} values were 11.25, 4.01 and 3.70 μM against MDA-MB231, MDA-MB468 and MCF7 cells lines, respectively. Compound **2f** with 2-hydroxyl and 3-methoxyl substitutions in the phenyl ring exhibited greater cytotoxic potency than the corresponding free carboxylic derivative **1d** or cisplatin. Surprisingly, Compound **2f**, the methyl ester analog of compound **1h** did not show any improved antiproliferative activity.

4. Conclusion

In conclusion, various substituted 2-(phenyl)-3*H*-Benzo[*d*]Imidazole-5-carboxylic acids (**1a–1j**) and its methyl esters (**2a–2f**) were prepared by condensing equimolar quantities of commercially available hydroxy benzaldehydes with 3-, 4-diamino benzoic acid and its methyl ester and then screened for their antiproliferative activity on breast cancer cell lines (MDA-MB231, MDA-MB468 and MCF7) *in vitro*. Almost 50% of the compounds synthesized showed either comparable to or greater potency than cisplatin, the compound that we used as a reference. Among the carboxylic acid derivatives, compound **1h** with 3, 4 dihydroxy substitution in the aryl ring is the most active with GI_{50} values of 12.85, 11.85 and 9.23 μM against MDA-MB468, MDA-MB231 and MCF7 breast cancer cells respectively. Among the methyl ester derivatives, compound **2e** with 2-hydroxyl and 5-fluoro substitution in the aryl ring was the most potent with GI_{50} values of 6.23, 4.09 and 0.18 μm against MDA-MB468, MDA-MB231 and MCF breast cancer cells respectively. Taken together, our data suggest that the title compounds particularly compounds **1h** and **2e**, can be promising lead compounds in the development of more effective cancer therapeutics for treatment of breast cancer.

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