Potential Anticancer Agents: Design, Synthesis of New Pyrido[1,2-a] benzimidazoles and Related Derivatives Linked to Alkytating Fragments

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Abstract

The incentive of the present work has been primarily directed towards the design and synthesis of some novel pyrido[1,2-a]benzimidazoles with specific functionalities believed to have alkylating ability. This combination of pharmacological agents may enable synergistic anticancer effect. Nine compounds 5b, 13a, 13d, 13e, 14b, 14c, 15, 16, and 17 were selected by the National Cancer Institute (NCI), Bethesda, Maryland, USA to be evaluated for their in vitro antitumor activity. All the selected compounds were tested initially at a single dose (10 µM) in the full NCI 60 cell panel including leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancer cell lines. Majority of the test compounds exhibited moderate cytotoxic activity. The highest activity in the investigated cancer cells was displayed by 14c against melanoma SK-MEL-5 cell line. This may be due to the impact of the lipophilic trifluoromethyl substitution on the biological activity profile.

Keywords: Design; Synthesis; Substituted pyridine; 2-pyridone; Antitumor activity

Introduction

Cancer is a devastating affliction, the frequency of which is progressively increasing all over the world. Its occurrence is escalating rapidly and is a major cause in health complications [1]. The treatment approach dictates that the treatment of cancer is directed toward eradication of all cancer cells and this is attained by frenziedly discovery of new candidates of anticancer activity [2].

Previously, we have utilized pyrido[1,2-a]benzimidazole (PBI) as a privileged scaffold for the design of many PBI derivatives of potential cytotoxic activity [3-8]. In fact, this ring system is characterized by the presence of pyridine or 2-pyridone units which constitute a subject of great interest due to their extensive presence in the skeletal backbone of many biologically active compounds. They possess a wide variety of biological activities such as antituber [9], antidiabetic [9], anti-inflammatory [10], anticoagulant [11], antiviral [12], antibacterial [13], antifungal [14] and anticancer activities [15]. Pyridine moiety is one of the building units of some tyrosine-kinase inhibitors; imatinib is used in the treatment of multiple cancers; whereas, sorafenib is used in the treatment of advanced renal and hepatocellular carcinoma [16]. The 2-pyridone unit is an integral part of some cytotoxic agents such as roquinimex which investigated as adjuvant therapy after bone marrow transplantation in chronic myelogenous leukemia [17] and diazaquinomycin A which demonstrates in vitro cytotoxicity against some tumor cell lines [18].

Among the investigated PBI series, NSC649900 [3], NSC682011 [4] and NSC699944 [5] (Figure 1) were identified by the NCI as promising candidates for further testing in an in vitro anticancer hollow fiber assay because of their good cytotoxic activity and subpanel disease selectivity especially against leukaemic cell in the in vitro screen. In fact, the PBI backbone of these compounds demonstrates structural complementarity with the isosteric β-carboline and pyrido[2,3-a] indolizine (PI) which constitute the key scaffolds of many cytotoxic agents such as the β-carboline alkaloid harmine which is identified as a useful inhibitor of tumor development [19] and the antitumor antibiotic camptothecin [20].

Biochemical data suggests that camptothecin act as DNA topoisomerase I inhibitor. It possesses a novel mechanism of action involving the inhibition of DNA relaxation by DNA topoisomerase I, and more specifically the stabilization of a covalent binary complex formed between topoisomerase I and DNA [20]. In addition, it is proposed that the planar nature of camptothecin allows its intercalation between DNA base pairs at the site of single-strand cleavage [21]. For this reason, it may be worthy to study the possible interactions of NSC649900, NSC682011 and NSC699944 with topoisomerase-I as a target enzyme because of the evident structural complementarity between PBI scaffold of these agents and pyrido[2,3-a]indolizine (PI) backbone of camptothecin (Figure 2). Docking results revealed that NSC649900, NSC682011 and NSC699944 displayed arene-arene interactions with one or more amino acid residues similar to camptothecin NSC649900 showed arene-arene interactions with DAC113 and TGBP11 residues, in addition to hydrogen bonding with ArgD364; whereas, NSC682011 displayed arene-arene interactions with TGBP11 and ArgD364.

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Previously, we have utilized pyrido[1,2-a]benzimidazole (PBI) as a privileged scaffold for the design of many PBI derivatives of potential cytotoxic activity [3-8]. In fact, this ring system is characterized by the presence of pyridine or 2-pyridone units which constitute a subject of great interest due to their extensive presence in the skeletal backbone of many biologically active compounds. They possess a wide variety of biological activities such as antituber [9], antidiabetic [9], anti-inflammatory [10], anticoagulant [11], antiviral [12], antibacterial [13], antifungal [14] and anticancer activities [15]. Pyridine moiety is one of the building units of some tyrosine-kinase inhibitors; imatinib is used in the treatment of multiple cancers; whereas, sorafenib is used in the treatment of advanced renal and hepatocellular carcinoma [16]. The 2-pyridone unit is an integral part of some cytotoxic agents such as roquinimex which investigated as adjuvant therapy after bone marrow transplantation in chronic myelogenous leukemia [17] and diazaquinomycin A which demonstrates in vitro cytotoxicity against some tumor cell lines [18].

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amino acid residues. In addition, NSC699944 revealed arene-arene interactions with TGPB11 and DAC113 amino acid residues. The result indicates that the planar PBI scaffold contributes to binding to the main active sites similar to camptothecin and it is possible that these PBIs may intercalate between DNA base pairs of topo I-DNA complex through arene-arene interactions (Figure 2). Inspired by these findings and in a continuation of our efforts to discover and explore new heterocyclic compounds of promising anticancer activities two series of PBIs comprising pyridine and 2-pyridone nuclei (Figure 3) were designed to be synthesized and evaluated for their \textit{in vitro} anticancer activity. Both series are designed with specific functionalities such as 2-hydroxalkyl, 2-chloroalkyl, piperidino- and morpholino alkyl moieties at position-2 through an aminomethylene spacer (Figure 3, Scheme 1) or at position-1(Figure 3, Scheme 2). Other analogues comprising aliphatic amino and aryl amino moieties are proposed.

Alkylating fragments such as 2-chloroethylamino and \textit{N},\textit{N}-bis(2-chloroethyl)amino and selected substituents such as 2-hydroxethylamino and morpholinoalkyl are incorporated in the PBI scaffold of the proposed compounds because of their importance in the backbone structure of some antineoplastic drugs; namely, Mitoxantrone [22], Lomustine [23], Bendamustine [24] and Gefitinib [25].

**Experimental**

**Chemistry**

All reagents and solvents were purchased from commercial suppliers and were purified and dried when necessary by standard techniques. Melting points were determined in open glass capillaries using Stuart capillary melting point apparatus (Stuart Scientific Stone, Staffordshire, UK) and are uncorrected. IR spectra were recorded, for potassium bromide discs, \( \nu \) (cm\(^{-1}\)), on Perkin Elmer 1430 spectrophotometer. \(^1\)H-NMR spectra were determined either on a Bruker Avance spectrometer (400 MHz) at the microanalytical unit, Faculty of Science, Cairo University, or on Jeol (125 MHz) at the microanalytical unit, Faculty of Science, Alexandria University, using DMSO-\(d_6\) as a solvent and TMS as internal standard. The chemical shifts are given in \( \delta \) ppm values (s, singlet; d, doublet; t, triplet and m, multiplet). \(^13\)C-NMR spectra were determined on Jeol (125 MHz), Faculty of Science, Alexandria University, using TMS as internal standard. Mass spectra were run on a Finnigan mass spectrometer model SSQ/7000 (70 eV), Faculty of Science, Cairo University. Microanalyses were performed at the microanalytical unit, Faculty of Science, Cairo University. The results of the microanalyses were within \( \pm 0.4\% \) of the calculated values. Follow-up of the reactions and checking the homogeneity of the compounds were made by ascending TLC run on silica gel G (Merck 60) coated glass plates. The spots were visualized by exposure to iodine vapor or UV lamp at \( \lambda \) 254 nm for few seconds.

Pyridobenzimidazole-4-carbonitrile was prepared according to a reported procedure in a good yield through cyclocondensation of 1H-benzimidazol-2-yl-acetonitrile with ethyl acetocetate in presence of ammonium acetate [7]. Formylation of pyridobenzimidazole-4-
carbonitrile was achieved by applying Vilsmeier Haack reaction by the addition of phosphorus oxychloride to a well stirred suspension of pyridobenzimidazole-4-carbonitrile to afford the aldehyde (1) [7], while 1-Chloro-3-methylpyridobenzimidazole-4-carbonitrile (10) was prepared by refluxing pyridobenzimidazole-4-carbonitrile in excess phosphorus oxychloride according to a reported procedure [7].

2-((tert-Butylamino)methylene-3-methyl-1-oxo-1,2-dihydropyrido[1,2-a]benzimidazole-4-carbonitrile (2): A mixture of 2-formyl-3-methyl-1-oxo-1,5-dihydropyridobenzimidazole-4-carbonitrile (1) (2 mmol, 0.50 g) and 2-methylpropan-2-amine (3 mmol, 0.22 g) in dimethylformamide (10 ml) was stirred at room temperature for 20 h. The reaction mixture was then diluted with ice cold water. The obtained product was filtered, washed with water, dried and crystallized from dimethylformamide/ethanol. Yield 69.63%, M.P.>300°C; IR (KBr, cm⁻¹): 3231 (NH), 3107, 3055, 3024, 2968, 2930, 2873 (C-H), 2218 (C=O), 1655 (C=O), 1614 (C=N), 1561 (C=C). 1H-NMR (400 MHz, DMSO-d₆) δ (ppm): 1.50 (s, 9H, 3 CH₃), 2.67 (s, 3H, CH₃ at C₃), 7.34 (t, J=7.64 Hz, 1H, pyridobenzimidazole C₇-H), 7.44 (t, J=7.64 Hz, 1H, pyridobenzimidazole C₈-H), 7.74 (d, J=7.98 Hz, 1H, pyridobenzimidazole C₆-H), 8.35 (d, J=14.65 Hz, 1H, methine H), 8.41 (d, J=7.97 Hz, 1H, pyridobenzimidazole C₉-H), 11.60 (d, J=14.23 Hz, 1H, NH, D₂O exchangeable). Anal. Calcd. for C₁₈H₁₈N₄O (306.37): C, 70.57; H, 5.92; N, 18.29. Found: C, 70.74; H, 5.97; N, 18.47.

2-((2-Hydroxyethyl)amino)methylene-3-methyl-1-oxo-1,2-dihydropyrido[1,2-a]benzimidazole-4-carbonitrile (3): A mixture of 2-formyl-3-methyl-1-oxo-1,5-dihydropyridobenzimidazole-4-carbonitrile (1) (2 mmol, 0.50 g) and 2-aminoethan-1-ol (3 mmol, 0.18 g) in dimethylformamide (10 ml) was stirred at 60-80°C for 4-6 h. The reaction mixture was then diluted with ice cold water. The obtained product was filtered, washed with water, dried and crystallized from...
dimethylformamide/ethanol. Yield 50.97%, M.P. 282°C; IR (KBr, cm \(^{-1}\)): 3463 (OH), 3250 (NH), 2227 (C≡N), 1649 (C=O), 1615 (C=N), 1562, 1448 (C=C). 1H-NMR (400 MHz, DMSO-d\(_6\)) δ (ppm): 2.59 (s, 3H, CH\(_3\)), 3.69 (t, J=7.72 Hz, 4H, 2 CH\(_2\)), 5.07 (t, J=4.53 Hz, 1H, OH, D\(_2\)O exchangeable), 7.33 (t, J=7.64 Hz, 1H, pyridobenzimidazole C\(_7\)-H), 7.44 (t, J=7.64 Hz, 1H, pyridobenzimidazole C\(_8\)-H), 7.73 (d, J=8.02 Hz, 1H, pyridobenzimidazole C\(_6\)-H), 8.40 (d, J=8.02 Hz, 1H, pyridobenzimidazole C\(_9\)-H), 8.46 (d, J=14.59 Hz, 1H, methine H), 11.16-11.33 (m, 1H, NH, D\(_2\)O exchangeable). 13C-NMR (125 MHz, DMSO-d\(_6\), δ ppm): 17.98 (CH\(_3\)), 53.12 (NCH\(_2\)), 60.14 (OCH\(_2\)), 87.10 (pyridobenzimidazole C\(_4\)), 98.38 (CN), 115.46 (pyridobenzimidazole C\(_9\)), 116.83 (pyridobenzimidazole C\(_2\)), 119.03, 122.91, 125.63 (pyridobenzimidazole C\(_6\), C\(_7\), C\(_8\)), 130.81 (pyridobenzimidazole C\(_9\)-a), 144.22 (pyridobenzimidazole C\(_5\)-a), 148.72 (pyridobenzimidazole C\(_4\)-a), 153.84 (pyridobenzimidazole C\(_3\)), 161.56 (pyridobenzimidazole C\(_1\)), 161.76 (methine CH). Anal. Calcd. for C\(_{16}\)H\(_{14}\)N\(_4\)O\(_2\) (294.31): C, 65.30; H, 4.79; N, 19.04. Found: C, 65.43; H, 4.85; N, 19.18.

2-[(2-Chloroethyl)amino]methylene-3-methyl-1-oxo-1,2-dihydropyrido[1,2-a]benzimidazole-4-carbonitrile (4): A suspension of 3 (2 mmol, 0.59 g) in phosphorous oxychloride (6 ml) was heated under reflux for 3 h while stirring. The reaction mixture was allowed to cool to room temperature and then poured onto crushed ice. The mixture was neutralized with Na\(_2\)CO\(_3\) and the obtained product was filtered, washed with water, dried and crystallized from DMF/ethanol. Yield 89.53%, M.P. >300°C; IR (KBr, cm \(^{-1}\)): 3231 (NH), 3091, 3018, 2962, 2935, 2870 (C-H), 2214 (C≡N), 1655 (C=O), 1610 (C=N), 1556 (C=C), 770 (C-Cl). 1H-NMR (400 MHz, DMSO-d\(_6\)) δ (ppm): 2.59 (s, 3H, CH\(_3\)), 3.91-4.06 (m, 4H, 2 CH\(_2\)), 7.34 (t, J=7.56 Hz, 1H, pyridobenzimidazole C\(_7\)-H), 7.44 (t, J=7.56 Hz, 1H, pyridobenzimidazole C\(_8\)-H), 7.73 (d, J=7.85 Hz, 1H, pyridobenzimidazole C\(_6\)-H), 8.36 (d, J=14.26 Hz, 1H, methine H), 8.42 (d, J=7.88 Hz, 1H, pyridobenzimidazole C\(_9\)-H), 11.61 (m, J=13.82 Hz, 1H, enamine NH, D\(_2\)O exchangeable). Anal. Calcd. for C\(_{16}\)H\(_{13}\)ClN\(_4\)O (312.76): C, 61.44; H, 4.19; N, 17.91. Found: C, 61.59; H, 4.24; N, 18.07.

3-Methyl-1-oxo-2-[(2-(substituted)ethyl]amino]methylene-1,2-dihydropyridobenzimidazole-4-carbonitrile (5a-c): A suspension of 2-[(2-chloroethyl)amino]methylene-3-methyl-1-oxo-1,2-dihydropyridobenzimidazole-4-carbonitrile (4) (2 mmol, 0.63 g) and the corresponding amine (6 mmol) in a mixture of absolute ethanol (15 ml) and dimethylformamide (6 ml) was stirred at room temperature for 10 h in case of compound (5a) and was refluxed for 10 h in case of both compounds (5b, c). Crushed ice was added to the reaction mixture and the obtained product was filtered, washed with water, dried and crystallized from the proper solvent.

2-[(2-Chloroethyl)amino]methylene-3-methyl-1-oxo-1,2-dihydropyridobenzimidazole-4-carbonitrile (5a-c): A suspension of 2-[(2-chloroethyl)amino]methylene-3-methyl-1-oxo-1,2-dihydropyridobenzimidazole-4-carbonitrile (4) (2 mmol, 0.63 g) and the corresponding amine (6 mmol) in a mixture of absolute ethanol (15 ml) and dimethylformamide (6 ml) was stirred at room temperature for 10 h in case of compound (5a) and was refluxed for 10 h in case of both compounds (5b, c). Crushed ice was added to the reaction mixture and the obtained product was filtered, washed with water, dried and crystallized from the proper solvent.

2-[(2-Chloroethyl)amino]methylene-3-methyl-1-oxo-1,2-dihydropyridobenzimidazole-4-carbonitrile (5a): Yield 51.43%, M.P.:>300°C; crystalization solvent: dioxane/ethanol; IR (KBr, cm \(^{-1}\)): 3419 (NH), 3058, 2969, 2935 (C-H), 1655 (C=O), 1615 (C=N), 1560 (C=C), 870 (pyridobenzimidazole C\(_4\)), 53.12 (NCH\(_2\)), 60.14 (OCH\(_2\)), 87.10 (pyridobenzimidazole C\(_4\)), 98.38 (CN), 115.46 (pyridobenzimidazole C\(_9\)), 116.83 (pyridobenzimidazole C\(_2\)), 119.03, 122.91, 125.63 (pyridobenzimidazole C\(_6\), C\(_7\), C\(_8\)), 130.81 (pyridobenzimidazole C\(_9\)), 144.22 (pyridobenzimidazole C\(_9\)), 148.72 (pyridobenzimidazole C\(_9\)), 153.84 (pyridobenzimidazole C\(_9\)), 161.56 (methine CH), 161.63 (pyridobenzimidazole C\(_9\)). Anal. Calcd. for C\(_{20}\)H\(_{23}\)N\(_5\)O (349.44): C, 68.74; H, 6.63; N, 20.04. Found: C, 68.98; H, 6.69; N, 20.21.

3-Methyl-1-oxo-2-[(2-(tert-Butylamino)ethyl]amino]methylene-3-methyl-1-oxo-1,2-dihydropyridobenzimidazole-4-carbonitrile (5a): Yield 51.43%, M.P.:>300°C; crystalization solvent: dioxane/ethanol; IR (KBr, cm \(^{-1}\)): 3419 (NH), 3058, 2969, 2935 (C-H), 1655 (C=O), 1615 (C=N), 1560 (C=C), 870 (pyridobenzimidazole C\(_4\)), 53.12 (NCH\(_2\)), 60.14 (OCH\(_2\)), 87.10 (pyridobenzimidazole C\(_4\)), 98.38 (CN), 115.46 (pyridobenzimidazole C\(_9\)), 116.83 (pyridobenzimidazole C\(_2\)), 119.03, 122.91, 125.63 (pyridobenzimidazole C\(_6\), C\(_7\), C\(_8\)), 130.81 (pyridobenzimidazole C\(_9\)), 144.22 (pyridobenzimidazole C\(_9\)), 148.72 (pyridobenzimidazole C\(_9\)), 153.84 (pyridobenzimidazole C\(_9\)), 161.56 (methine CH), 161.63 (pyridobenzimidazole C\(_9\)). Anal. Calcd. for C\(_{20}\)H\(_{23}\)N\(_5\)O (349.44): C, 68.74; H, 6.63; N, 20.04. Found: C, 68.98; H, 6.69; N, 20.21.
MHz, DMSO-d6 δ (ppm): 1.40-1.53 (m, 6H, CH3), 2.44-2.50 (m, 6H, CH2-N and piperidine C3,4-H2), 2.60 (s, 3H, CH3), 3.71 (m, 2H, CH2-N), 7.33 (t, J=7.64 Hz, 1H, pyridobenzimidazole C6-H), 7.43 (t, J=7.64 Hz, 1H, pyridobenzimidazole C7-H), 7.72 (d, J=7.93 Hz, 1H, pyridobenzimidazole C8-H), 8.39 (d, J=8.01 Hz, 1H, pyridobenzimidazole C9-H), 8.46 (d, J=14.13 Hz, 1H, methine H), 11.18 (m, 1H, NH, D2O exchangeable). Anal. Calcld. for C18H17ClN4O (340.81): C, 63.64; H, 5.03; N, 16.44. Found: C, 63.59; H, 5.11; N, 16.72.

2-[1-Chloro-2-methylprop-2-yl]amino)methylene-3-methyl-1-oxo-1,2-dihydropyridine (4): A suspension of 2-methylpyrido[1,2-a]benzimidazole-4-carbonitrile (1) (2 mmol, 0.50 g) and 2-amino-2-methylpropan-1-ol (3 mmol, 0.27 g) in dimethylformamide (10 ml) was stirred at 60-80°C for 4-6 h. The mixture was then diluted with ice cold water. The obtained product was filtered, washed with water, dried and crystallized from dimethylformamide/ethanol. Yield 17.56%, M.P. 244°C; IR (KBr, cm-1): 3413 (NH), 3256 (NH), 2923, 2872 (C-H), 2210 (C≡N), 1653, 1599 (C=C). Anal. Calcld. for C18H16N4O3 (338.37): C, 63.89; H, 5.36; N, 16.43. Found: C, 63.92; H, 5.40; N, 16.42.

2-[1-Chloro-2-methylprop-2-yl]amino)methylene-3-methyl-1-oxo-1,2-dihydropyridine (5): A mixture of 4-carbonitrile (1) (4 mmol, 0.97 g) and ethanolamine (12 mmol, 0.73 g) in dioxane (20 ml) was heated under reflux for 10 h. The reaction mixture was then poured into ice cold water. The product was filtered, washed with water, dried and crystallized from DMF/ethanol. Yield 93.46%, M.P. 286°C; IR (KBr, cm-1): 3423 (OH), 3256 (NH), 2923, 2872 (C-H), 2210 (C≡N), 1653, 1599 (C=C). Anal. Calcld. for C18H16N4O3 (338.37): C, 63.89; H, 5.36; N, 16.43. Found: C, 63.92; H, 5.40; N, 16.42.
3-Methyl-1-[2-(morpholin-4-yl)ethyl]aminopyrido[1,2-a]benzimidazole-4-carbonitrile (13d):

Yield 47.76%, M.P. = 258°C; crystallization solvent: dioxane. IR (KBr, cm⁻¹): 3377 (NH), 3070, 2975, 2834, 2803 (C-H), 2201 (C≡N), 1629 (C=N), 1596, 1583, 1458 (C=C); 1H-NMR (400 MHz, DMSO-d₆) δ (ppm): 2.51 (s, 3H, CH₃), 2.55 (t, J=4.44 Hz, 4H, morpholine CH₂-C=H), 2.76 (t, J=6.30 Hz, 2H, N-CH₂), 3.54 (q, J=6.2 Hz, 2H, CH₂-NH), 3.64 (t, J=4.49 Hz, 4H, morpholine CH₂-C=H), 6.13 (s, 1H, pyridobenzimidazole CH=H), 7.27 (t, J=4.06 Hz, 1H, N=O exchangeable), 7.36 (t, J=7.66 Hz, 1H, pyridobenzimidazole C=H), 7.53 (t, J=7.66 Hz, 1H, pyridobenzimidazole C=H), 7.8 (d, J=8.02 Hz, 1H, pyridobenzimidazole CH=H), 8.28 (d, J=8.34 Hz, 1H, pyridobenzimidazole C=H). Anal. Calcld. for C₂₇H₂₃N₄O (341.41): C, 73.60; H, 6.31; N, 21.06.

3-Methyl-1-[2-(piperazin-1-yl)ethyl]aminopyrido[1,2-a]benzimidazole-4-carbonitrile (13e):

Yield 74.63%, M.P. = 276°C; crystallization solvent: DMF/H₂O. IR (KBr, cm⁻¹): 3387, 3206 (NH), 3073, 2949, 2890, 2814 (C-H), 2200 (C≡N), 1630 (C≡N), 1596, 1561, 1458 (C=C). 1H-NMR (400 MHz, DMSO-d₆) δ (ppm): 2.52 (s, 3H, CH₃), 2.57-2.68 (m, 4H, piperazine CH₂-C=H), 2.77 (t, J=6.62 Hz, 2H, N-CH₂), 2.93 (t, J=4.66 Hz, 4H, piperazine CH₂-C=H), 3.53 (t, J=6.23 Hz, 2H, CH₂-NH), 6.13 (s, 1H, pyridobenzimidazole CH=H), 7.34 (t, J=7.72 Hz, 1H, pyridobenzimidazole C=H), 7.35 (t, J=7.72 Hz, 1H, pyridobenzimidazole C=H), 7.8 (d, J=8.05 Hz, 1H, pyridobenzimidazole CH=H), 8.3 (d, J=8.29 Hz, 1H, pyridobenzimidazole C=H). Anal. Calcld. for C₂₇H₂₃N₄O (343.43): C, 68.24; H, 6.63; N, 25.13. Found: C, 68.32; H, 6.68; N, 25.37.

3-Methyl-1-[4-(substituted phenylamino)pyrido[1,2-a]benzimidazole-4-carbonitrile (14a-c):

A mixture of the chloro derivative (10) (4 mmol, 0.97 g) and the proper arylamines, (12 (2 mmol, 0.57 g) and dimethylamine, tert-butylamine, piperidine, morpholine or piperazine (6 mmol) respectively, in 10.8 mixture of dioxane/dimethylformamide (18 ml) was stirred at room temperature for 24 hrs in case of compounds (13a,b) or at or 60-80°C for 12 hrs in case of compounds (13c-e). Crushed ice was added to the reaction mixture and the separated product was filtered, washed with water and crystallized from the proper solvent.

1-[2-(Dimethylamino)ethyl]amino-3-methylpyrido[1,2-a]benzimidazole-4-carbonitrile (13a):

Yield 37.29%, M.P.=258°C; crystallization solvent: DMF/ethanol. IR (KBr, cm⁻¹): 3382 (NH), 3036, 3010, 2978, 2950, 2816, 2816 (C-H), 2205 (C≡N), 1632 (C≡N), 1598, 1559, 1452, 1431 (C=C). 1H-NMR (400 MHz, DMSO-d₆) δ (ppm): 2.30 (s, 6H, 2 CH₃), 2.53 (s, 3H, CH₃ at C₄), 2.69 (t, J=6.29 Hz, 2H, N-CH₂), 3.52 (t, J=6.25 Hz, CH₂-NH), 6.15 (s, 1H, pyridobenzimidazole CH=H), 7.28 (s, 1H, NH, N=O exchangeable), 7.34 (t, J=7.80 Hz, 1H, pyridobenzimidazole C=H), 7.79 (d, J=8.32 Hz, 1H, pyridobenzimidazole CH=H), 8.23 (d, J=8.37 Hz, 1H, pyridobenzimidazole C=H). Anal. Calcld. for C₁₆H₁₃N₄O (353.37): C, 69.60; H, 6.53; N, 23.87. Found: C, 69.87; H, 6.59; N, 24.12.

1-[2-(tert-Butylamino)ethyl]amino-3-methylpyrido[1,2-a]benzimidazole-4-carbonitrile (13b):

Yield 78.13%, M.P.=286°C; crystallization solvent: ethanol. IR (KBr, cm⁻¹): 3536 (NH), 3302, 3113, 2966 (C-H), 2210 (C≡N), 1631 (C≡N), 1595 (C=C). 1H-NMR (400 MHz, DMSO-d₆) δ (ppm): 1.21 (s, 9H, 3 CH₃), 2.54 (s, 3H, CH₃ at C₄), 2.90-3.11 (m, 2H, N-CH₂), 3.49-3.73 (m, 2H, CH₂-NH), 6.25 (s, 1H, pyridobenzimidazole CH=H), 7.32 (t, J=7.47 Hz, 1H, pyridobenzimidazole C=H), 7.53 (t, J=7.47 Hz, 1H, pyridobenzimidazole CH=H), 7.79 (d, J=8.05 Hz, 1H, pyridobenzimidazole CH=H), 8.45 (d, J=8.18 Hz, 1H, pyridobenzimidazole C=H). Anal. Calcld. for C₂₉H₂₁N₄O (363.35): C, 71.00; H, 7.21; N, 21.79. Found: C, 71.24; H, 7.28; N, 21.88.

3-Methyl-1-[2-[(piperidin-1-yl)ethyl]aminopyrido[1,2-a]benzimidazole-4-carbonitrile (13c):

Yield 89.55%, M.P.=264°C; crystallization solvent: dioxane/ethanol. IR (KBr, cm⁻¹): 3360 (NH), 3041, 3102, 2975, 2931, 2853, 2829 (C-H), 2205 (C≡N), 1633 (C≡N), 1598, 1549, 1463 (C=C). 1H-NMR (400 MHz, DMSO-d₆) δ (ppm): 1.39-1.51 (m, 2H, piperidine C₄-H₆), 1.51-1.64 (m, 4H, piperidine C₄-H₄), 2.50 (under DMSO, 7H, CH₂ and piperidine C₄-H₂), 2.72 (t, J=6.31 Hz, 2H, ethyl N-CH₃), 3.51 (t, J=6.07 Hz, 2H, N-CH₂), 6.10 (s, 1H, pyridobenzimidazole CH=H), 7.28-7.39 (m, 2H, pyridobenzimidazole C=H and NH, N=O exchangeable), 7.53 (t, 1H, J=7.69 Hz, pyridobenzimidazole CH=H), 7.8 (d, J=8.00 Hz, 1H, pyridobenzimidazole C=H), 8.26 (d, J=8.25 Hz, 1H, pyridobenzimidazole CH=H).EI-Mass spectrum m/z (relative abundance%) 334.25 (91); 333.25 (3.25); 235.14 (1.64); 206.10 (1.03); 194.09 (0.87); 102.06 (0.79); 99.16 (6.71); 98.11 (100.00); 96.10 (1.72); 83.12 (0.85); 70.93 (8.32); 55.07 (2.03). Anal. Calcld. for C₂₇H₂₃N₄O (341.44): C, 72.04; H, 6.95; N, 21.00. Found: C, 72.18; H, 7.02; N, 21.17.
C2-H, 7.04 (d, J=8.5 Hz, 2H, methoxyphenyl C3-H), 7.29-7.36 (m, 3H, pyridobenzimidazole C3-H and methoxyphenyl C3-H), 7.54 (t, J=7.64 Hz, 1H of pyridobenzimidazole C7-H), 7.81 (d, J=6.75 Hz, 1H, pyridobenzimidazole C8-H), 8.48 (d, J=7.43 Hz, 1H, pyridobenzimidazole C9-H), 9.2 (s, 1H, NH, D2O exchangeable).

13C-NMR (125 MHz, DMSO-d6, δ ppm): 21.02 (CH3 at C3), 55.73 (OCH3), 41.12, 53.05, 73.24; H, 4.98; N, 17.14.

IR (KBr, cm-1): 3469 (NH), 2211 (C≡N), 1548, 1484 (C=C). 1H-NMR (400 MHz,DMSO-d6) δ (ppm): 2.58 (s, 3H, CH3), 6.59 (s, 1H, pyridobenzimidazole C3-H), 7.28-7.85 (m, 7H, pyridobenzimidazole C7-9-H and methoxyphenyl C1-3-H), 7.61 (d, J=7.80 Hz, 1H, pyridobenzimidazole C8-H), 9.69 (s, 1H, NH, D2O exchangeable). Anal. Calcd. for C17H16Cl2N4 (347.24): C, 58.80; H, 4.64; N, 16.14. Found: C, 58.79; H, 4.61; N, 16.32.

**Antitumor activity**

Nine compounds 5b, 13a, 13d, 13e, 14b, 14c, 15, 16, and 17 were selected by the National Cancer Institute (NCI), Bethesda, Maryland, USA to be evaluated for their *in vitro* antitumor activity. Effective one-dose assay has been added to the NCI 60 Cell screen in order to increase compound throughput and reduce data turnaround time to suppliers while maintaining efficient identification of active compounds. All the selected compounds were tested initially at a single dose (10 µM) in the full NCI 60 cell panel including leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancer cell lines. The results are presented in Table 1 as growth (%G) and only compounds which satisfy pre-determined threshold inhibition criteria would progress to the five-dose screen [26-29].

**Results and Discussion**

**Chemistry**

The synthetic procedures implemented to obtain the newly synthesized compounds are demonstrated in Schemes 1 and 2.

In Scheme 1, the enamine tautomers of Schiff bases 2, 3, 6 and 8 were synthesized by reacting the aldehyde 1 [7] with the proper amine. 1HNMR spectrum of compound 2 displayed a singlet at 1.50 ppm due to tert-butyl protons and a doublet at 8.35 ppm integrated for one proton attributed to the methine proton. It also revealed one D2O exchangeable singlet at 4.83 ppm characteristic for NH proton. Meanwhile, the 1HNMR spectrum of compound 3 revealed one triplet at 3.69 ppm attributed to the four protons of the ethanolamine side chain and one D2O exchangeable triplet at 5.07 ppm due to OH proton. One doublet at 8.46 ppm integrated for one proton representing the methine proton. Also its 13CNMR spectrum showed peaks at around 53 and 60 ppm corresponding to NCH2 and OCH2 moieties of the ethanolamine side chain. 1HNMR spectrum of compound 6 revealed a singlet at 4.42 ppm due to the six protons of the two CH2 groups of the side chain and a doublet at 3.50 ppm corresponding to the CH2-O protons. In addition, a D2O exchangeable triplet at 5.53 ppm due to OH proton was observed. The spectrum also showed one doublet at 8.31 ppm attributed to the methine proton and a doublet at 11.68 ppm characteristic for the NH proton. Additionally, 1HNMR spectrum of compound 8 revealed a singlet at 1.36 ppm due to the CH3 protons of the side chain and a D2O exchangeable triplet at 5.41 ppm due to two OH protons. Two doublets at 8.32 and 11.70 ppm, each integrated for one proton attributed to the methine and the NH protons, respectively were shown.

Chlorination of aliphatic OH group was achieved by heating the starting material 3, 6 and 8 in excess phosphorus oxychloride to give the corresponding chlorinated compounds 4, 7 and 9. The 1HNMR spectrum of compound 4 lacked the D2O exchangeable triplet of the OH proton and revealed a doublet at 8.52 ppm attributed to the OH proton and neutralized with Na2CO3. The product was filtered, washed with water and crystallized from dioxiane/ ethanol. Yield 89.93%, M. 237°C; IR (KBr, cm-1): 3055, 3020, 2961, 2920, 2874 (C=O), 2214 (C≡N), 1624 (C=N), 1593, 1511, 1442 (C=C). 1HNMR (400 MHz, DMSO-d6) δ (ppm): 2.60 (s, 3H, CH3), 3.63-3.96 (m, 8H, 2 NCH2 and 2 CH2-O), 4.56 (t, J=4.40 Hz, 2H, 2 OCH2). 6.69 (s, 1H, pyridobenzimidazole C3-H), 7.34 (t, J=7.69 Hz, 1H, pyridobenzimidazole C7-H), 7.52 (t, J=7.69 Hz, 1H, pyridobenzimidazole C8-H), 7.81 (d, J=8.06 Hz, 1H, pyridobenzimidazole C9-H). Anal. Calcd. for C17H16Cl2N4 (347.24): C, 58.80; H, 4.64; N, 16.14. Found: C, 58.97; H, 4.61; N, 16.32.
methine proton. The spectrum also showed a multiplet at around 11.2 ppm characteristic for the NH proton. Furthermore, the 1HNMR spectrum displayed peaks at 44.15 and 52.02 ppm attributed to NCH3 and CH2Cl moieties of the side chain, respectively. Nucleophilic displacement of aliphatic chlorine atom by aliphatic amines was achieved by reacting a suspension of 4 and the proper amine in a mixture of absolute ethanol and dimethylformamide to furnish compounds 5a-c. Their IR spectrum showed absorption bands between 3227-3420 cm⁻¹ corresponding to the NH groups. 1HNMR spectrum of compound 5a revealed a singlet at 1.50 ppm representing the nine protons of the tert-butyl group and one doublet at 8.86 ppm corresponding to the methine proton. A multiplet at 11.61 ppm attributed to the enamine NH proton was also displayed, whereas, 1HNMR spectrum of compound 5b revealed that it possesses a weak activity (G%) for PBI 31 (79.55%); prostate cancer PC-3 (88.18%) and breast cancer MCF7 (86.58%) and methylpyridobenzimidazole-4-carbonitrile (IMVI) (87.18%); melanoma SK-MEL-5 (89.05%) and UACC-62 (81.60%); colon cancer HT29 (88.74%) and U251 (85.61%) showed two multiplets due to the CH2Cl groups.

Table 1: The growth percentage (G %) in single-dose assay for the selected compounds.

| Comp No. | Leukemia | Non-Small Cell Lung Cancer | Colon Cancer | CNS Cancer | Melanoma | Ovarian Cancer | Renal Cancer | PC | Breast Cancer |
|----------|-----------|----------------------------|--------------|------------|-----------|---------------|--------------|----|-------------|
|          |          |                            |              |            |           |               |              |    |             |
| HL-60   |           |                            |              |            |           |               |              |    |             |
| (T8)    | RPMI-1786 | A549/ATCC                  | NCI-H460     | HCT-116    | HT29      | SNB-19        | ZU25         | LOX| SK-OV-3     |
|         |           |                            | NCI-H622     |            |           |               |              |    |             |
| 5b      | 90.61     | 96.51                      | 97.75        | 90.76      | 93.55     | 95.44         | 98.19        | 92.41| 97.03       |
| 13a     | 91.62     | 92.00                      | 96.43        | 87.18      | 97.86     | 97.20         | 101.65       | 90.98| 94.77       |
| 13d     | 97.26     | 92.67                      | 83.42        | 93.43      | 72.54     | 90.60         | 89.72        | 115.47| 72.77       |
| 15e     | 91.60     | 95.78                      | 87.59        | 84.71      | 85.52     | 75.01         | 84.65        | 100.01| 83.41       |
| 14b     | 43.83     | 93.43                      | 72.80        | 79.96      | 82.00     | 77.94         | 98.85        | 98.11| 96.65       |
| 14c     | 31.80     | 22.82                      | 43.73        | 7.23       | 2.32      | 20.69         | 39.61        | 13.19| 20.91       |
| 15      | 101.30    | 103.96                     | 97.24        | 96.90      | 97.98     | 101.40        | 109.59       | 95.59| 90.68       |
| 16      | 85.53     | 85.36                      | 94.77        | 90.03      | 83.88     | 22.15         | 86.43        | 92.78| 79.85       |
| 17      | 113.20    | 95.29                      | 66.70        | 80.34      | 69.49     | 75.33         | 83.41        | 111.75| 82.99       |

In vitro antitumor activity

Nine compounds 5b, 13a, 13d, 13e, 14b, 14c, 15, 16, and 17 were selected by NCI and tested initially at a single dose (10 μM) in the full NCI 60 cell panel line. The results are recorded as percentage growth (G%); for example, a value of (100%) means no growth inhibition. A value of (20%) would mean (80%) growth inhibition.

Results obtained for 5b revealed that it possesses a weak activity against NCI SNB-75 (83.72%); renal cancer CAKI-1 (89.03%) and UO-31 (79.55%); prostate cancer PC-3 (88.18%) and breast cancer MCF7 (86.44%) and T-47D (86.26%) cell lines.

The PBI's with substituted aminoethyl moiety at position-1 (13a, 13d and 13e) showed weak activity as indicated from (G%) for some cell lines; for compound 13a: non-small cell lung cancer HOP-62 (87.18%); melanoma SK-MEL-5 (89.05%) and UACC-62 (81.60%); renal cancer CAKI-1 (89.91%) and UO-31 (80.97%) and breast cancer MDA-MB-231/ATCC (84.44%) and T-47D (88.55%) cell lines. The (G%) for PBI (13d): non-small cell lung cancer A549/80CC (83.42%) and NCI-H522 (72.54%); renal cancer CAKI-1 (85.61%) and UO-31 (82.36%); and breast cancer MCF7 (88.74%), BT-549 (87.01%)
Compound 5b revealed a weak inhibitory effect against many cell lines from some types of cancer. This would indicate that the presence of 2-pyridone scaffold did not result in a significant improvement on the activity. Structural activity correlation revealed that the PBIs which lack the 2-pyridone unit but have instead a pyridine moiety showed variable cytotoxic activity. For instance, substitution at position-1 with N,N-dimethylaminoethylnylamino (13a), morpholinoethylnylamino (13d) and piperazinylythylamino (13e) did not let significant activity. On the other hand, the PBI which carry 4-methoxyphenylamino moiety (14b) exhibited weak inhibitory effects against several cell lines from non-small cell lung cancer, melanoma, renal and breast cancer and it demonstrated remarkable cytotoxic activity against one cell line from leukemia. It is worthy to mention that the highest anticancer activity was recorded for compounds 14c. Results revealed that replacement of the 4-methoxy (14b) with 3-trifluoromethyl group (14c) resulted in broad spectrum and variable degree of activity against many of the tested cell lines. In fact, this finding would indicate the impact of the lipophilic 3-trifluoromethylphenylamino substituents on the activity.

The presence of bis-(2-hydroxyethyl)aminogroup (16) did not show any significant impact on the activity; whereas, the PBI (17) which carry an alkylating fragment, bis-(2-chloroethylnyl)amino, displayed a remarkable inhibitory effect against colon cancer HCT-116 cell line and weak inhibitory effects against many cell lines from leukemia, melanoma, non-small lung cancer and colon, renal, prostate and breast cancer.

Conclusion

In conclusion, two series of benzimidazoles comprising pyridine and 2-pyridone nuclei together with various functionalities believed to have alkylating ability were synthesized. These series were designed as an example of a new molecular hybrids having anticancer activity. The anticancer activity results revealed that among the tested compounds, compound (14c) was found to possess promising anticancer activity and the most significant inhibition as revealed from the growth percentage (G%) was found against melanoma SK-MEL-5 (4.24%) and UACC-62 (11.90%) and breast cancer BT-549 (19.39%) and T-47D (8.68%) cell lines. In fact, this finding together with the remarkable antineoplastic activity reported to the related PBI analogues (NSC682011 and NSC699944, Figure 1) would indicate the impact of the lipophilic 3-trifluoromethylphenylamino and 4-fluorophenylamino substituents on the activity. Although, none of the screened compounds satisfied the threshold inhibition criteria to pass for evaluation in the full panel five-dose in vitro antitumor screen, The PBI 14c can be considered starting structure that merit further optimization in order to design more active lead compounds for further studies.

Declaration of Interest

The authors declared no conflicts of interest. Only the authors are responsible for the content and writing of the paper.

References

1. Fujisawa D, Inoguchi H, Shimoda H, Yoshiuchi K, Inoue S, et al. (2016) Impact of depression on health utility value in cancer patients. Psychooncology 25: 491-495.
2. Mathew B, Hobrah JV, Connelly MC, Kipling Guy R, Reynolds RC (2017) Diverse amide analogs of aulindac for cancer treatment and prevention. Bioorg Med Chem Lett 15: 27: 4614-4621.
3. Badawy EAM, Kapp T (1995) Benzimidazole condensed ring system. IX. Potential antineoplastics. New synthesis of some pyrindol[1,2-d]benzimidazoles and related derivative. Eur J Med Chem 30: 327-332.
4. Badawey EAM, Kappe T (1999) Benzimidazole condensed ring systems. XI. Synthesis of some substituted cycloalkyl pyrido [1,2-a] benzimidazoles with anticipated antineoplastic activity. Eur J Med Chem 34: 663-667.

5. El-Hawash SAM, Badawey EAM, Kappe T (1999) Benzimidazole condensed ring systems. XII. Synthesis and anticancer evaluation of certain pyrido [1,2-a] benzimidazole derivatives. Pharmazie 54: 341-346.

6. Badawey EAM, Rida SM, Soliman FSG, Kappe T (1999) Benzimidazole condensed ring systems. III. Synthesis of some substituted 2,3-dihydrocyclopenta-1H-[4',5'-2,3]pyrido[1,2-a]benzimidazole-11-carbonitrides. Monatsh Chem 120: 73-76.

7. Rida SM, Soliman FSG, Badawey EAM, Kappe T (1999) Benzimidazole condensed ring systems. XII. Synthesis and anticancer evaluation of certain pyrido [1,2-a] benzimidazole derivatives. Pharmazie 54: 341-346.

8. Badawey EAM, Rida SM, Soliman FSG, Kappe T (1995) Benzimidazole condensed ring systems 10 (1). Synthesis and Cytotoxic activity of some pyrido [1,2-a] benzimidazole-4-carbonitriles and related derivatives. J Heterocyclic Chem 25: 1725-1729.

9. Baumann M, Baxendale IR (2013) An overview of the synthetic routes to the best-selling drugs containing 6-membered heterocycles. Beilstein J Org Chem 9: 2265-2319.

10. Sweetman SC (2009) Martindale. The Complete Drug Reference. 36th edn. Pharmaceutical Press, pp: 117-177.

11. Parlow JJ, Kurumbail RG, Stegemann RA, Stevens AM, Stallings WC, et al. (2003) Design, synthesis, and crystal structure of selective 2-pyridone derivatives as potential antimicrobial agents. J Med Chem 46: 4696-4701.

12. Sweetman SC (2009) Martindale. The Complete Drug Reference. 36th edn. Pharmaceutical Press, pp: 882-884.

13. Desai NC, Shihory NR, Kotadiya GM (2014) Facile synthesis of benzimidazole bearing 2-pyridones and related derivatives as potential antimicrobial agents. Chinese Chemical Letters 25: 305-307.

14. Gupta AK, Pott T (2004) Ciclopirox: a broad spectrum antifungal with antibacterial and anti-inflammatory properties. International Journal of Dermatology 43: 3-8.

15. Hamilton G, Ostrowski U (2013) Picoplatin pharmacokinetics and chemotherapy of non-small cell lung cancer. Expert Opin Drug Metab Toxicol 9: 1381-1390.

16. Sweetman SC (2009) Martindale. The Complete Drug Reference. 36th edn. Pharmaceutical Press, pp: 733-770.

17. Isaacs JT (2010) The long and winding road for the development of tasquinimod as an oral second-generation quinoline-3-carboxamide antiangiogenic drug for the treatment of prostate cancer. Expert Opin Investig Drugs 19: 1235-1243.

18. Maskey RP, Grun-Wollny I, Laatsch H (2005) Isolation and structure elucidation of Diazaquinomycin C from a terrestrial streptomycetes sp. and confirmation of the akashin structure. Natural Product Research 19: 137-142.

19. Jimenez J (2008) Cytoxicity of the β-carboline alkaloids harmine and harmaline in human cell assays in vitro. Experimental and Toxicologic Pathology 60: 381-389.

20. Thomas CJ, Rahier NJ, Hecht SM (2004) Camptothecin: current perspectives. Bioorganic & Medicinal Chemistry 12: 1565-1604.

21. Staker BL, Feese MD, Cushman M (2005) Structures of three classes of anticancer agents bound to the human topoisomerase I- DNA covalent complex. J Med Chem 48: 2336-2345.

22. Dogra S, Awasthi P, Tripathi S, Pradeep TP, Nair MS, et al. (2014) NMR-based structure of anticancer drug mitoxantrone stacked with terminal base pair of DNA hexamer sequence d-(ATCGAT)2. J Biolom Strud Dyn 32: 1164-1163.

23. Tanis JB, Mason SL, Maddox TW, Blackwood L, Killick DR, et al. (2018) Evaluation of a multi-agent chemotherapy protocol combining lomustine, procarbazine and prednisolone (LPP) for the treatment of relapsed canine non-Hodgkin high-grade lymphomas. Vet Comp Oncol.

24. Keating MJ, Bach C, Yasothon U, Kirpatrick P (2008) Bendamustine. Nat Rev Drug Discov 7: 473-474.

25. Sweetman SC (2009) Martindale. The Complete Drug Reference. 36th edn. Pharmaceutical Press, pp: 754-755.

26. Alley MC, Scudiere DA, Monks A, Hursey ML, Czerwinski MJ, et al. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Research 48: 589-601.

27. Boyd MR, Paull KD (1995) Some practical considerations and applications of the National Cancer Institute: Cancer drug discovery and development program. Drug Development Research 34: 91-109.

28. Grever MR, Schepartz SA, Chabner BA (1992) The National Cancer Institute: Cancer drug discovery and development program. Semin Oncol 19: 622-638.

29. Shoemaker RH (2007) The NCI60 human tumour cell line anticancer drug screen. Nature Reviews 6: 813-823.