Synthesis and Pharmacologic Evaluation of Some Benzimidazole Acetohydrazide Derivatives as EGFR Inhibitors

EGFR İhbitörü Olarak Bazı Asetohidrazit Türevi Benzimidazollerin Sentezi ve Farmakolojik Değerlendirilmesi

Serkan DEMİREL¹, Gülgün AYHAN KILÇIĞİL*¹, Zümra KARA², Berna GÜVEN², Arzu ONAY BEŞİKCI²

¹Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Ankara, Turkey
²Ankara University, Faculty of Pharmacy, Department of Pharmacology, Ankara, Turkey

OBJECTIVES: In this study, some novel 2-(2-phenyl)-1H-benzo[d]imidazol-1-yl)-N’-(arylmethylene) acetohydrazide derivatives (1-12) were designed and synthesized.

MATERIALS AND METHODS: Compounds 1-12 were obtained by condensing 2-(2-phenyl)-1H-benzo[d]imidazol-1-yl)acetohydrazide (III) with the corresponding aromatic aldehyde derivatives in the presence of catalytic amounts of hydrochloric acid in ethanol.

RESULTS: Following the structure elucidation, epidermal growth factor receptor kinase inhibitor activity was measured. The ADP-Glo™ kinase assay determines kinase activity based on the quantification of the amount of ADP produced during a kinase reaction.

CONCLUSION: Almost all of the compounds’ kinase inhibitor activities were rather limited.

KEY WORDS: Benzimidazole, acetohydrazide, epidermal growth factor receptor kinase, inhibitory

INTRODUCTION

Cancer is a lethal disease characterized by an uncontrolled proliferation of cells, invasive nature, and metastasis. In developed countries, following cardiovascular diseases, it is the second most common cause of death. According to research conducted worldwide in 2015, 8.8 million people died of cancer, which is nearly 1 in 6 of all deaths. With standard therapies, cancer is often not completely removed and new treatments are sought.

Tyrosine kinase belongs to the protein kinase family and it provides for protein phosphorylation. Phosphorylation of proteins through kinases plays an important role in signal transduction mechanisms. Changes that occur in signal transduction play an important role in cancer development and metastases because it controls functions such as cell proliferation and apoptosis. Epidermal growth factor receptor (EGFR), the first cell surface receptor associated with cancer, is a tyrosine kinase receptor. EGFR is involved in cancer development and metastasis.
Studies have shown that inhibition of EGFR can arrest or slow the development of cancer. EGFRs have been observed to be overproduced in lung, breast, prostate, bladder, ovarian, colon, and rectum cancers, and they are found to be structural in most normal epithelial cells (e.g., skin, hair follicles). Thus, inhibition of EGFR activity inhibits cell proliferation and leads to apoptosis, programmed cell death. EGFR, such as other tyrosine kinase receptors, may also be inhibited by both monoclonal antibodies and tyrosine kinase inhibitors. Other inhibitors of tyrosine kinase such as gefitinib, erlotinib, lapatinib are small molecules that target EGFR and are used in cancer therapy. It is known that compounds used as inhibitors of tyrosine kinase such as sunitinib carry benzimidazole isostere indole rings, and pazopanib and axitinib have indazole rings. Furthermore, by determining that benzimidazoles exhibit protein kinase inhibitor activity, in addition to their different biologic activities, the compounds planned to be synthesized in this study are thought to be directly related to cancer development.

We designed and synthesized new benzimidazole acetohydrazide derivatives bearing a phenyl ring at the 2nd position and N-aryl methylideneacetohydrazide (1-12) (Table 1) at the 1st position, and evaluated their EGFR kinase inhibitory activities by comparing them with erlotinib. Following the structure elucidation, EGFR kinase inhibitor activities of the synthesized derivatives were measured (Table 1).

**EXPERIMENTAL**

**General synthetic**

All starting materials and chemical reagents used in the synthesis were high-grade commercial products purchased from Aldrich or Merck (Germany). Analytical thin-layer chromatography was performed using Merck precoated TLC plates, and spots were visualized with ultraviolet light. Melting points were determined using a Thermo Scientific Electrothermal IA9100 digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK) and were uncorrected. The structures of all synthesized compounds were assigned on the basis of nuclear magnetic resonance (NMR) and mass spectral analyses. 1H-NMR spectra were recorded on a Varian Mercury 400 MHz instrument (Varian Inc., Palo Alto, CA, USA) using a tetramethylsilane internal standard and DMSO-d6; coupling constants (J) are reported in Hertz. All chemical shifts are reported as δ (ppm) values. ESI-MS were obtained using a Waters QW Micromass LC-MS spectrometer (Waters Corporation, Milford, MA, USA) with the positive electrospray ionization method. All instrumental analyses were performed at the Central Instrumentation Laboratory of the Pharmacy Faculty of Ankara University, Ankara, Turkey.

**General procedure for the preparation of 2-(2-phenyl)-1H-benzimidazol-1-yl)-N′- (arylmethylidene) acetohydrazide derivatives (1-12)**

Aromatic aldehyde derivative (0.02 mol) was added to a solution of the acyl hydrazide III (0.02 mol) in absolute EtOH (5 mL) containing a catalytic amount of 37% hydrochloric acid. The mixture was refluxed for 60 min, poured into cold water, and neutralized with 10% aqueous sodium bicarbonate solution. Crude product was filtered off and crystallized from dimethylformamide (DMF) water.

| Compounds | Ar                  | Inhibition % |
|-----------|---------------------|--------------|
| 1         | 4-chlorophenyl       | 8.63         |
| 2         | 4-fluorophenyl       | 1.93         |
| 3         | 4-bromophenyl        | 8.63         |
| 4         | 3-nitrophenyl        | 13.71        |
| 5         | 2-naphthyl           | 0            |
| 6         | 3-methylthiophene    | 0            |
| 7         | 4-benzylxylophenyl   | 12.42        |
| 8         | 2-chloro-5-nitrophenyl| 0            |
| 9         | 3,4-dibenzylxylophenyl| 9.92     |
| 10        | 3-bromo-4-fluorophenyl| 0            |
| 11        | 2,4-dichlorophenyl   | 7.69         |
| 12        | 4-chloro-3-nitrophenyl| 3.01         |

Erlotinib

1 μM: 90.06

100 nM: 74.56

M.P: 267°C; 1H NMR δ 5.06 and 5.52 (2s, 2H, CH2), 7.25-7.28 (m, 2H, Ar-H), 7.48-7.57 (m, 6H, Ar-H), 7.70-7.75 (m, 5H, Ar-H), 8.03 and 8.24 (2s, 1H, CH=N), 11.88 (brs, 1H, NH); ESI-MS (m/z): 389.4 (100%) (M+H), 391.5 (40%) (M+2+H).

M.P: 214-217°C; 1H NMR δ 5.05 and 5.51 (2s, 2H, CH2), 7.24-7.29 (m, 4H, Ar-H), 7.52-7.57 (m, 4H, Ar-H), 7.70-7.80 (m, 5H, Ar-H), 8.04 and 8.24 (2s, 1H, CH=N), 11.79, 11.93 (2s, 1H, NH); ESI-MS (m/z): 373.36 (M+H).

M.P: 287°C; 1H NMR δ 5.06 and 5.52 (2s, 2H, CH2), 7.25-7.30 (m, 2H, Ar-H), 7.52-7.80 (m, 11H, Ar-H), 8.02 and 8.22 (2s, 1H, CH=N), 11.86 (brs, 1H, NH); ESI-MS (m/z): 433.38 (100%) (M+H), 435.34 (95%) (M+2+H).

M.P: 280°C; 1H NMR δ 5.09 and 5.57 (2s, 2H, CH2), 7.25-7.29 (m, 2H, Ar-H), 7.51-7.59 (m, 4H, Ar-H), 7.69-7.80 (m, 4H, Ar-H), 8.17, 8.52 (m, 3H, Ar-H), 8.53 and 8.55 (2s, 1H, CH=N), 12.04, 12.20 (2s, 1H, NH); ESI-MS (m/z): 400.5 (M+H).
**RESULTS AND DISCUSSION**

**Chemistry**

The benzimidazole acetohydrazide derivatives were synthesized according to the following synthetic scheme: 2-Phenyl-1H-benzo[d]imidazole (I) was prepared via the reaction of o-phenylenediamine, benzaldehyde and sodium metabisulfite in DMF. The reaction of I with ethyl chloroacetate yielded ethyl 2-(2-phenyl)-1H-benzo[d]imidazol-1-yl) acetate (II). Treatment of the ester (II) with hydrazine hydrate gave the desired hydrazide compound, 2-(2-phenyl)-1H-benzo[d]imidazol-1-yl) acetohydrazide (III). Compounds 1-12 were found by condensing acyl hydrazide III with the corresponding aromatic aldehydes in the presence of sulfuric acid (Scheme 1). The structure of the newly synthesized compounds was confirmed and supported by spectroscopic data. The mass spectrum of each of compound according to the following synthetic scheme: 2-Phenyl-1H-benzo[d]imidazole (I) was prepared via the reaction of o-phenylenediamine, benzaldehyde and sodium metabisulfite in DMF. The reaction of I with ethyl chloroacetate yielded ethyl 2-(2-phenyl)-1H-benzo[d]imidazol-1-yl) acetate (II). Treatment of the ester (II) with hydrazine hydrate gave the desired hydrazide compound, 2-(2-phenyl)-1H-benzo[d]imidazol-1-yl) acetohydrazide (III). Compounds 1-12 were found by condensing acyl hydrazide III with the corresponding aromatic aldehydes in the presence of sulfuric acid (Scheme 1). The structure of the newly synthesized compounds was confirmed and supported by spectroscopic data. The mass spectrum of each of compound...
appeared as M+H peak, consistent with the molecular formula of the assigned structure. N-acylhydrazones may be present as four isomers depending on the geometric isomerism relative to the imino group (E, Z isomers) and conformers related to the amide linkage (syn/anti amide conformers).14 In 1H-NMR spectra measured in polar solvent dimethyl sulfoxide-d<sub>6</sub>, two signals representing the imino hydrogen (CH=N) were observed at 7.98-8.61 and 8.18-8.67 ppm, the methylene protons (CH<sub>2</sub>CO) were also typically observed as two singlet signals at 5.01-5.10 and 5.19-5.58 ppm, respectively.

Biological activities

The ADP-Glo™ kinase assay<sup>15</sup> determines kinase activity based on the quantification of the amount of ADP produced during a kinase reaction. EGFR is a tyrosine kinase receptor and was the first receptor to be proposed for cancer therapy. Currently, there are two approaches that target EGFRs in cancer treatment: monoclonal antibodies and small-molecule inhibitors of EGFR tyrosine kinase enzymatic activity. Erlotinib selectively inhibits tyrosine kinase activity of EGFRs.

In the present study, we measured the activity of EGFR kinase and evaluated the inhibitory efficiencies of newly synthesized compounds by comparing them with erlotinib. The assays were performed in three steps (Figure 1). A kinase reaction with 5 ng EGFR kinase and 50 µM ATP was the first step. The amounts of both EGFR and ATP were determined in the preliminary optimization assays (results not shown). The total volume of the kinase reaction was 25 µL. The amount of the EGFR and PolyE<sub>4</sub>Y<sub>1</sub> substrate was 1 µg, based on the recommendations of the literature. In the second step, an equal volume of ADP-Glo<sup>TM</sup> reagent was added to terminate the kinase reaction and deplete the remaining ATP. On the third and final step, a kinase detection reagent was added to convert the ADP produced during the kinase reaction to ATP and to determine the amount of newly synthesized ATP through a luciferase/luciferin reaction.

Inhibitory efficiency was determined by comparing the enzyme activities in the presence of the inhibitors with the maximum enzyme activity. Maximum kinase activity (100% kinase) was determined as the luminescence acquired in the kinase reaction where EGFR kinase, PolyE<sub>4</sub>Y<sub>1</sub> ATP and diluting agent, and 5% DMSO were present in the reaction medium.

CONCLUSION

In the reactions where inhibitory efficiencies were determined, the reaction medium contained either erlotinib or one of the newly synthesized compounds in a single concentration of 1 µM. At this concentration, erlotinib inhibited EGFR kinase activity by nearly 90%. Inhibitory efficiencies of the new compounds are shown in Table 1. The results indicated that almost all of the compounds’ kinase inhibitor activities were somewhat limited. Compounds that have 3-nitrophenyl (4) and 4-benzyloxyphenyl (7) substituents as aryl groups were found to have slight inhibitory potencies in the kinase assay at 13.71% and 12.42%, respectively.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. http://www.who.int/cancer
2. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298:1912-1934.
3. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006;366:2-16.

4. Bianco R, Gelardi T, Damiano V, Ciardiello F, Tortora G. Rational bases for the development of EGFR inhibitors for cancer treatment. Int J Biochem Cell Biol. 2007;39:1416-1431.

5. Lin Y, Wang X, Jin H. EGFR-TKI resistance in NSCLC patients: mechanisms and strategies. Am J Cancer Res. 2014;4:411-435.

6. Hasegawa M, Nishigaki N, Washio Y, Kano K, Harris PA, Sato H, Mori I, West RL, Shibahara M, Toyoda H, Wang L, Nolte RT, Veal JM, Cheung M. Discovery of novel benzimidazoles as potent inhibitors of TIE-2 and VEGFR-2 tyrosine kinase receptors. J Med Chem. 2007;50:4453-4470.

7. Li Y, Tan C, Gao C, Zhang C, Luan X, Chen X, Liu H, Chen Y, Jiang Y. Discovery of benzimidazole derivatives as novel multi-target EGFR, VEGFR-2 and PDGFR kinase inhibitors. Bio Med Chem. 2011;19:4529-4535.

8. Yadav S, Sinha D, Singh SK, Singh VK. Novel benzimidazole analogs as inhibitors of EGFR tyrosine kinase. Chem Biol Drug Des. 2012;80:625-630.

9. Abdel-Ghaffar NF. Synthesis, reactions, structure-activity relationship of 2-benzimidazole analogs as anticancer agents and study their molecular docking. Der Pharma Chem. 2013;243-257.

10. Ridley HF, Spickett RGW, Timmis GM. A new synthesis of benzimidazoles and aza analogs. J Heterocyclic Chem. 1965;2:453-456.

11. Heaney H, Ley SV. N-Alkylation of indole and pyroles in dimethyl sulfoxide. J Chem Soc Perkin I. 1973;499-500.

12. Smith PAS. In: Adams R, Bachmann WE, Fieser LF, Johnson JR, Snyder HR. (Eds). Organic Reactions. John Wiley & Sons, Inc. London; Chapman & Hall, Limited; 1949;3:337-389.

13. Carvalho SA, Feitosa LO, Soares M, Costa TE, Henriques MG, Salomão K, de Castro SL, Kaiser M, Brun R, Wardell JL, Wardell SM, Trossini GH, Andricopulo AD, da Silva EF, Fraga CA. Design and synthesis of new (E)-cinnamic N-acylhydrazones as potent antitrypanosomal agents. Eur J Med Chem. 2012;54:512-521.

14. Patorski P, Wyrzykiewicz E, Bartkowiak G. Synthesis and Conformational Assignment of N-(E)-Stilbenloxymethylenecarbonyl-Substituted Hydrazones of Acetone and o-(m- and p-) Chloro- (nitro-) benzaldehydes by Means of 1H and 13C NMR Spectroscopy. J Spectrosc. 2013;2013:1-12.

15. ADP-Glo™ Kinase Assay. Technical Manual. Promega.