Synthesis and cytotoxicity studies of 1-propenyl-1,3-dihydro-benzimidazol-2-one

Biswadip Banerji¹ · Sumit Kumar Pramanik¹

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Abstract A heterocyclic compound 1-propenyl-1,3-dihydro-benzimidazol-2-one was synthesized by a palladium-catalyzed rearrangement reaction. Anticancer activities were confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against Neura 2a (neuroblastoma cell), HEK 293 (kidney cancer) and MCF-7 (breast cancer) cell lines at low micromolar range. Furthermore, clear images from phase-contrast and fluorescence microscopes and confocal images unambiguously confirm the cancer cell death. The single X-ray crystal structure of the compound unambiguously proves the structure of the benzimidazolone compound.

Keywords Benzimidazolone · Palladium catalyzed · MTT assay · Confocal microasopy

Introduction

Worldwide cancer is one of the most common and fatal diseases that cause deaths [1, 2]. The existing anticancer drugs are not sufficient enough to combat this serious problem [3, 4]. Hence, identification of new, selective molecules which have low toxicity and their easy synthesis remain a challenging task

for the cancer therapy [5–7]. Benzimidazolone scaffold is one of the key scaffolds of a variety of heterocyclic compounds that play crucial roles in the modulation of a number of biologically important pathways [8–11]. Thus, benzimidazole and its derivatives representing a major class of nitrogen-containing heterocycles have gained an important role in the drug discovery [12–15]. The biological importance of benzimidazole derivatives is due to their structural resemblance to the naturally occurring nucleotides. This has allowed them to interact with the biopolymers of the living system [16]. Substituted benzimidazole derivatives have lots of therapeutic applications in the areas including antimicrobial [17], antioxidant [18], antiviral [19], antihypertensive [20], antiprotozoal [21], anti-inflammatory [22], and molluscicidal [23] agents. Many benzimidazole derivatives were reported to selectively inhibit endothelial cell growth and suppress angiogenesis both in vitro and in vivo [24]. Some potential anticancer activities were also reported with compounds having benzimidazolone scaffold [25]. Such characteristics have made this scaffold a synthetic target, and therefore, its synthesis and derivatization become an important and challenging job.

In an ongoing project in our laboratory on the synthesis of “new chemical entity” (NCE) having anticancer activity, we were interested to synthesize benzimidazolone-derived anticancer molecule, and screen them against different cancer cell lines. A palladium-catalyzed rearrangement reaction was employed for the synthesis of this scaffold. The cell viability was performed by MTT assay against Neura 2a (neuroblastoma cell), HEK 293 (kidney cancer), and MCF-7 (breast cancer) cell lines. The anticancer property of these molecules was further investigated by taking images from inverted phase-contrast microscope, fluorescent microscope, and confocal studies. The images indicated that cell death might have occurred through mitochondrial membrane dysfunction.

CCDC-776996 contains the supplementary crystallographic data for this paper. This data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.Ccdc.cam.ac.uk/data_request/cif

✉ Biswadip Banerji
biswadip.banerji@gmail.com

¹ Department of Chemistry, CSIR-Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata 700032, India

Methods and materials

Chemistry

o-Phenylenediamine, *trans*-crotonyl chloride, Pd(PPh₃)₂Cl₂, xantphos, and NaO^tBu were purchased from Acros Organics (Geel, Belgium). All the solvents and the common lab reagents were purchased from Fischer Chemicals. Flash chromatography was performed on silica gel (Merck, 100–200 mesh) as the stationary phase. All ¹H and ¹³C NMR spectra were recorded on a Bruker 600 MHz spectrometer. For ¹H NMR, tetramethylsilane (TMS) served as internal standard (*d*=0), and data are reported as follows: chemical shift, integration, multiplicity (singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m)), and coupling constant(s) in hertz. For ¹³C NMR, TMS (*d*=0) or CDCl₃ (*d*=77.26) was used as internal standard, and spectra were obtained with complete proton decoupling. Mass spectra were obtained on a JEOL MStation 700. IR spectra were recorded on a JASCO FT/IR-4200 spectrometer.

Reaction protocol to synthesize

1-propenyl-1,3-dihydro-benzimidazol-2-one

To a mixture of amide **3** (0.228 mmol) and NaO^tBu (0.458 mmol) in dry toluene (4 ml) in a round-bottom flask, Pd(PPh₃)₂Cl₂ (0.0228 mmol) was added, followed by xantphos (0.0458 mmol) before degassed with nitrogen for 2 min, and finally, the reaction mixture was stirred for 3 h at 80 °C. After this period, it was cooled to room temperature and filtered through celite pad, and the filtrate was concentrated under reduced pressure. The resulting residue was finally purified by flash chromatography (silica gel, petroleum ether/EtOAc) to afford the desired product **4** (yield 67 %).

1-propenyl-1,3-dihydro-benzimidazol-2-one (**4**)

White solid, *R*_f≈0.50 (petroleum ether/EtOAc, 3:1); melting point: 150 °C; ¹H NMR (600 MHz, CDCl₃): δ (in ppm) 1.91 (*d*, 3H, CH₃, *J*=8.4), 6.14–6.20 (*m*, 1H, Ar), 6.70 (*d*, 1H, CH, *J*=15.6), 7.08–7.13 (3H, CH, and Ar, *m*), 7.25 (*d*, 1H, Ar, *J*=7.2), 9.80 (exch br s, 1H, NH); ¹³C NMR (150 MHz, CDCl₃): δ (in ppm) 15.86, 109.27, 109.75, 117.21, 121.37, 121.57,

122.15, 127.81, 129.04, 154.32; HRMS (EI+): *m/z* Calcd for C₁₀H₁₀N₂O (M)⁺ 174.0793, found: *m/z* 174.0802; FTIR (γ/cm⁻¹, KBr): 3150, 3061, 2960, 1713, 1663, 1618, 1484, 1397, 1273, 1187, 1097, 937.

Biology

Cell culture

Neuroblastoma cell lines Neuro 2a, breast cancer cell line MCF-7, and human embryonic kidney cell lines HEK 293 were procured from the National Centre for Cell Sciences (NCCS; Pune, India) and were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Life Technologies, USA) supplemented with 10 % fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin and gentamicin). Cells were cultured at 37 °C in 95 % air and 5 % CO₂ humidified incubators. MCF-7 cells were seeded at a density of 10⁵/well plated in 96-well plates. Cells were typically grown to 60–70 % confluence and then rinsed in phosphate-buffered saline (PBS). After that, they are placed into a serum-free medium for overnight prior to the treatments. After overnight incubation, the MCF-7 cells, HEK 293 cells, and Neuro 2a cells were treated with compound **4** (1-propenyl-1,3-dihydro-benzimidazol-2-one) at the concentration of 1, 10, and 20 μM. After 24 h, the medium was discarded and 50 μl of fresh medium was added along with 10 μl of MTT (5 mg/ml). MTT solution was slowly removed after 4 h, and the purple crystals were solubilized in 1.5 ml of DMSO. The absorbance was measured at test wavelength of 550 nm in ELISA Plate Reader made of DYNEX Technologies, USA.

JC-1 staining

JC-1 dye was purchased from Invitrogen (Life Technologies, Grand Island, NY, USA). HEK 293 cells were treated with 1-propenyl-1,3-dihydro-benzimidazol-2-one for overnight and then stained with JC-1 dye for checking mitochondrial health. The cells were then imaged under a fluorescence microscope. Cells with proper mitochondrial health show punctuated red staining along with green scattered staining, whereas the cells with mitochondrial dysfunction show only scattered green staining without any red puncta.

Scheme 1 a TEA, DCM, 0 °C. b Pd(PPh₃)₂Cl₂, xantphos, NaO^tBu, dry toluene, 80 °C

Fig. 1 The proposed mechanism of the formation of the benzimidazolone compound **4**

Active caspase 3 staining

Active caspase 3 antibody was purchased from Abcam, USA. Here, the HEK 293 cells were treated with 1-propenyl-1,3-dihydro-benzimidazol-2-one for overnight. Here, the cells were fixed with 4 % paraformaldehyde (PFA) and immunostained using primary antibody against active caspase 3 and Alexa Fluor 546 as secondary antibody. The cells were imaged under a confocal microscope.

Results and discussion

A palladium-catalyzed rearrangement protocol was developed to synthesize N-substituted benzimidazolone derivative [1-propenyl-1,3-dihydro-benzimidazol-2-one] (**4**) as shown in Scheme 1.

Crotonyl amide **3** was synthesized by using the previously reported protocol [26]. Crotonyl amide **3** was treated with $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ (5 mol%), xantphos (10 mol%) in dry toluene using NaO^tBu as base to get a rearranged product, **4** in 67 % yield. It was observed that in compound **4**, the double bond has migrated from the carbonyl to the ring nitrogen. A similar result was obtained with $\text{Pd}(\text{OAc})_2$ catalyst (yield 58 %). Other palladium catalysts used in this reaction were $\text{Pd}(\text{dppf})\text{Cl}_2$, $\text{Pd}(\text{PPh}_3)_4$, and $\text{Pd}_2(\text{dba})_3$, but they all turned out to be low yielding and sluggish in nature. Next, the base was changed from NaO^tBu to Cs_2CO_3 , and only 10 % conversion after 12 h was observed, showing that strong base was required for the reaction. Hence, with $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ and NaO^tBu combination, different ligands were screened to get

the optimized reaction condition. Monodentate phosphines like $\text{P}(\text{o-Tol})_3$, P^tBu_3 , HBF_4 , and DPPP [1,3-bis(diphenylphosphino)propane] ligands were not good for the reaction as only trace product was observed (<10 % yield), whereas good to moderate yield was obtained with 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP) and BDTBP ligands. Under the optimized reaction conditions, maximum conversion to the benzimidazolone **4** was achieved in 4 h of reaction time. After considerable experimentation, it was found that reaction proceeded extremely slowly in the room temperature (<5 % conversion in 12 h) and that heating at 80 °C was necessary for the rearrangement to proceed within 4 h. The final compound was recrystallized from the solution of the mixture of dichloromethane-hexane solvent. According to the proposed mechanism, the reaction may be conceived as follows: the π -Pd complex (I) first undergoes insertion reaction with the aromatic amine to produce (II). This intermediate (II) then undergoes a rearrangement via intermediate III to produce the final compound **4** as shown in the Fig. 1. The single X-ray crystal structure of **4** unambiguously proves the *trans*-geometry of the newly rearranged double bond in the product.

From the single crystal diffraction data analysis, it is confirmed that the molecule **4** is crystallized as monoclinic P21/n space group. Each of them accommodates four asymmetric molecules per unit cell. For the sake of brevity, the perspective view of the molecule in thermal ellipsoids drawn at 40 % probability levels is depicted in Fig. 2(a). In the Fig. 2, the intermolecular hydrogen bonding interaction is observed between O1 and N1 proton of adjacent molecule. These two atoms are like donor and acceptor. Hydrogen bonding interaction is 1.993 Å which is much less.

Fig. 2 **a** Structure of the asymmetric unit of molecule with thermal ellipsoids drawn at the 40 % probability level. **b** Intermolecular hydrogen bonding interaction

Fig. 3 Cytotoxicity studies against Neuro 2a, MCF-7, and HEK 293 cell lines

The benzimidazolone compound, **4**, showed cytotoxicity and killed different cultured cancer cell lines. Neuroblastoma cell lines Neuro 2a, breast cancer cell line MCF-7, and human embryonic kidney cell lines HEK 293 were subjected to 1-propenyl-1,3-dihydro-benzimidazol-2-one at different concentrations. The viable cells were evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay where the viability of the cells was determined by the reduction of the yellow MTT into the purple formazan product by mitochondrial dehydrogenase present in metabolically active cells [27]. The absorbance obtained from treated cells were expressed as percentages of absorbance obtained from untreated cells and are reported as mean $\pm$ Sd ($N=3$) [where Sd is Standard deviation and N is the number of experiment done]. Here, Fig. 3 shows the cell viability of different cell lines after incubation of the control and with 1, 10, and 20 μ M concentration of compound **4**.

It is found that the proliferation of these cancer cells was reduced at a dose of 20 μ M after 24 h. After incubation, the

cell viability was measured and estimated to be 50.76, 37.67, and 47.08 % for the Neuro 2a, MCF-7, and HEK 293, respectively, at 20 μ M concentration of compound **4**. To confirm the cytotoxicity of benzimidazolone compound, the treated cells were also examined under an inverted phase-contrast microscope. For example, HEK 293 cells were incubated with compound **4** for 24 h, and then phase-contrast microscopy images were taken. As shown in Fig. 4, there was substantial cell death on the addition of compound **4** as compared to the control cell lines.

In maximum cases, the cell death occurs through the disorder of active mitochondria [28]. This mitochondrial disruption includes changes in the membrane potential and alterations to the oxidation–reduction potential of the mitochondria [29]. Changes in the membrane potential are supposed to be due to the opening of the mitochondrial permeability evolution pore, allowing channel of ions and small molecules. In order to study mitochondrial membrane potential, JC-1 dye was widely used in fluorescent microscopy for a variety of cell types, including myocytes and neurons, as well as in intact tissues and isolated mitochondria. Cells with good mitochondrial health show punctuated red staining along with green scattered staining, whereas the cells with mitochondrial dysfunction show only scattered green staining without any red punctuation [30]. To check the efficacy of this molecule, cells were treated with compound **4**. For this purpose, HEK 293 cells were treated with the compound **4** at 20 μ M concentration for 24 h. After that, the cells were stained with JC-1 dye (10 mg ml⁻¹) for 10 min at 37 °C and the live cell imaging was performed under a fluorescence microscope.

Figure 5 shows that the cells treated with benzimidazolone compound resulted in the loss of mitochondrial membrane potential as punctuated red staining had diminished as compared to control. This result indicates that benzimidazolone compound might have caused cell death by mitochondrial dysfunction.

The caspase family of cysteine proteases play a key role in apoptosis and inflammation. Active caspase 3 proteolytically cleaves and activates other caspases, as well as relevant targets in the cells [31]. Here, we wish to check the effect of this

Fig. 4 Phase-contrast images showing cell death

Fig. 5 HEK 293 cells were treated with benzimidazolone compound for overnight then stained with JC-1 dye for checking mitochondrial health. The cells were then studied and imaged under a fluorescence microscope. *First and second rows*, respectively, show control cells and cells treated with benzimidazolone. The *first column* represents green fluorescent monomer of JC-1, the *second column* represents reddish orange fluorescence of J-aggregate, and the *third column* shows merged images. The scale bar represents 25 μm

benzimidazolone compound on activate caspase 3. First, HEK 293 cells with 20 μM of this benzimidazolone compound for 24 h. After that, the cells were fixed by 4 % PFA and fixed cells were immunostained by using a primary antibody which is specific for cleaved caspase 3, and Alexa Fluor 546 was used as a secondary antibody. After that, the cells were imaged under confocal microscope. The outcome showed elevated active caspase 3 levels in treated cells compared to that of control cell lines (Fig. 6) indicating cell death.

Fig. 6 HEK 293 cells were treated with benzimidazolone compound for overnight. The cells were fixed with 4 % paraformaldehyde (PFA) and immunostained using primary antibody against active caspase 3 and Alexa Fluor 546 as secondary antibody. The cells were imaged under a confocal microscope. *First and second rows* show control cells and cells treated with benzimidazolone compound, respectively. *First and second columns* show red staining for active caspase 3 and phase-contrast micrographs of cells, respectively. The scale bar represents 20 μm

Conclusion

The development of new anticancer drug is a challenging job, and particularly, the success rate is quite low because of its complexity. Thus, the synthesis and cytotoxicity study of new molecules with novel architecture as selective anticancer agent remain the major thrust in research. In this communication, the synthesis of a palladium-catalyzed rearranged molecule 1-propenyl-1,3-dihydro-benzimidazol-2-one has been reported. In vitro cytotoxicity studies of this molecule against

Neura 2a, MCF-7, and HEK 293 cell lines show low micromolar range anticancer activity. Furthermore, the cell death was confirmed by images from phase-contrast, fluorescence, and confocal microscope.

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