Synthesis and characterization of some benzidine-based azomethine derivatives with molecular docking studies and anticancer activities

Musa Erdoğan¹ · Ali Yeşildağ² · Barış Yıldız² · Burak Tüzün⁴ · Özkan Özden²

Received: 20 February 2023 / Accepted: 14 July 2023 / Published online: 25 July 2023
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Abstract
In this study, a benzidine-based azomethine derivat 2 with a proposed new mechanism and its two derivatives 4a-b have been designed, synthesized and characterized by ¹H, ¹³C NMR, FT-IR, and HRMS spectroscopic techniques, and their anticancer properties were investigated. The target compounds 2, 4a-b were obtained with excellent yields (91% and above) by condensation of benzidine (1) with three different aldehyde derivatives (formaldehyde, benzaldehyde 3a or p-nitrobenzaldehyde 3b) in refluxing EtOH. Surprisingly, treatment of benzidine (1) with formaldehyde afforded N,N,N,N'-tetrakis(ethoxymethyl)-[1,1'-biphenyl]-4,4'-diamine (2). The anticancer properties of these benzidine derivatives 2, 4a-b against two cell lines (MDA-MB-231 human breast adenocarcinoma and DLD1 human colorectal adenocarcinoma cell lines) were investigated with a colorimetric assay using the tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salts). The obtained results showed that the benzidine-based azomethine derivatives 2, 4a-b had a significant effect on the human breast cancer cell line (MDA-MB-231). Then, molecular docking calculations were made to compare the biological activities of benzidine-based azomethine derivatives 2, 4a-b against cancer proteins. ADME/T analysis was performed to examine the drug properties of benzidine-based azomethine derivatives 2, 4a-b. The compounds 2, 4a-b are promising as potential anticancer drug candidates.
Keywords Azomethine derivatives · Anticancer activities · MDA-MB-231 · DLD1 · Molecular docking · ADME/T

Introduction

Cancer refers to a collection of more than 200 different types of cancer disease. Cancer occurs by a series of mutations in specific genes in a cell, particularly controlling cell growth and division. This cell divides without stopping and spreads into surrounding tissues. Cancer is the second leading cause of mortality worldwide, and breast and colorectal cancers constitute the cancer types with the greatest frequency after lung cancer (Ashour et al. 2020; Muskinja et al. 2019). There are many types of cancer treatment, including surgery, radiotherapy, and systemic therapy (Riaz et al. 2019). Chemotherapy is one of the most common treatment methods for cancer and may be used in combination with other methods, such as surgery and radiation therapy. Since chemotherapeutic agents used to remove a tumor in a specific location of the body circulates throughout the body, they can carry a risk of side effects whose severity depends on the drug. Synthesizing novel chemotherapeutic agents having strong anticancer potential with minimum side effects is still a high necessity (Farghaly et al. 2020; Ngameni et al. 2021).

Benzidine (4,4’-diaminobiphenyl) is an aromatic amine compound containing a biphenyl scaffold and is a biologically and pharmacologically important skeleton. Benzidine derivatives have been widely used for the detection of blood and, as a starting material in dye production. Benzidine-based azo dyes are important and are widely used in industry (Amer et al. 2020).

A color additive is a chemical compound that reacts with another substance and causes color formation. The pharmaceutical industry uses various inorganic and organic dyes. The use of dyes in the pharmaceutical industry has commercial, psychological and practical purposes. Different drug colors can help patients distinguish different strengths of the same drug, reducing the risk of overdose or underdose (Šuleková et al. 2017). Most dyes/colorants used in the pharmaceutical industry are considered to belong to one of the following groups: azo dyes, quinoline dyes, triphenylmethane dyes and xanthine dyes (Pawar and Kumar 2002). Azo compounds represent about two-thirds of all synthetic dyes. They are the most widely used and structurally diverse classes of organic dyes in the trade. Their chemical formula is \( R–N = N–R \), where \( –N = N– \) represents the azo group and...
R or R' is aryl or alkyl compound (Chung 2016; Erdoğan et al. 2023; Yeşildağ et al. 2021). The azo dyes frequently used in pharmaceutical production are: E102 Tartrazine, E110 Sunset Yellow FCF, Ponceau 4R (Cochineal Red A), Azorubine (Karmoisine), Amaranth, E133 Brilliant Blue and E129 Allura Red. Many azo dyes like a benzidine show carcinogenic and mutagenic activity and may cause allergic reactions. In general, the toxicity of the components increases with the increase in benzene rings in their structure. The carcinogenicity of azo dyes directly depends on the structure of the molecule and the degradation mechanism. The degradation products of azo dyes are mostly aromatic amines with different structures and may also have carcinogenic properties (Haley 1975). Some azo dyes can be carcinogenic without cleavage to aromatic amines. However, the carcinogenicity of many azo dyes is due to their decomposed products such as benzidine. Benzidine induces a variety of human and animal tumors. Another azo dye component, p-phenylenediamine, is a contact allergen. Reduction of azo dyes can be accomplished by human intestinal microflora, skin microflora, environmental microorganisms, to a lesser extent human liver azoreductase, and non-biological means (Prival et al. 1984). Besides the side effects of these compounds, they are used in many diverse fields of industry.

Compounds containing azomethine groups are very stable and easily synthesized in a one-step condensation reaction between aldehydes and amines (Qin et al. 2013; Xin and Yuan 2012). Besides, they are also known as environmentally friendly compounds because water is the only byproduct in these reactions and complex purification is not required (Petrus et al. 2014). Moreover, such compounds have increased interest in these compounds due to their wide use in industry, in many fields of chemistry, medicine and pharmaceutical industry, making them widely used in different fields (Krzyzsztof et al. 2013). Among these applications, it came to the fore as a bioactive substance due to the contribution of the azomethine group to bioactivity by interacting with certain regions in the cell structure and forming hydrogen bonds (Venugopala and Jayashree 2003). The azomethine derivatives are biologically active compounds in medicinal and biological applications as antibacterial (Anush et al. 2018; Cleiton et al. 2011; Joseph et al. 2013; Lapasam et al. 2019), antimalarial (Dhar and Taploo 1982; Harpstrite et al. 2008; Rathelot et al. 1995), and anticancer (Miri et al. 2013; Modi et al. 1970; Uddin et al. 2020), agents over the past few years. It has been reported in many studies that azomethine derivatives obtained from the reaction of various amines and aldehydes have anticancer activity.

It is seen in recent studies that theoretical studies are increasing their popularity quickly and reliably. Due to the developments in technology and the high accuracy of the results obtained from theoretical studies, theoretical studies have become very common (Gürdere et al. 2021; Gezegen et al. 2021; Bilgiçi et al. 2021). There are many programs and methods used in theoretical calculations. The best-known today is molecular docking calculations. In this study, cancer proteins used against benzidine-based azomethine derivatives 2, 4a-b: protein structure of MLK4 kinase domain (pdb ID: 4UYA) (Marusiak et al. 2016) and crystal structure of the BRCT repeat region from the breast cancer associated protein (pdb ID: 1JNX) (Williams et al. 2001).

The MLK4 protein used in this study demonstrates its tumor suppressor role, and kinases are tumor suppressors in many tumor types, including colon, lung, pancreatic and ovarian cancers (Kennedy and Davis 2003; Whitmarsh and Davis 2007). The C-terminal BRCT region of BRCA1, another protein, is a protein required for DNA repair, transcription regulation, and tumor suppressor functions (Miki et al. 1994). There are two BRCT repeats that adopt similar structures in the BRCT region of human BRCA1 and are packaged together in an end-to-end arrangement (Futreal et al. 1994; Friedman et al. 1994). False mutations that cause breast cancer occur at the interface between the two repeats and destabilize the structure. After molecular docking calculations, ADME/T calculations were made to examine the properties of benzidine-based azomethine derivatives 2, 4a-b as drugs.

Herein, we successfully synthesized in high-yield three benzidine-based azomethine derivatives 2, 4a-b which were synthesized simply in a one-step condensation reaction between aldehydes (formaldehyde, benzaldehyde 3a or p-nitrobenzaldehyde 3b) and amine (benzidine 1). According to our best knowledge, one of these compounds (compound 2) has been undetermined in the literature except for one of our studies which reports its photovoltaic properties (Yeşildağ et al. 2022), and the other two compounds (the compounds 4a-b) have not been studied in human colon cancer cells or human breast cancer cells as anticancer agents so far. Moreover, the chemical structure of the synthesized compounds was determined by NMR, FT-IR, and HRMS. The anticancer activities of these benzidine-based azomethine derivatives 2, 4a-b against human colon cancer cells (DLD1) and human breast cancer cells (MDA-MB-231) were investigated. The synthesized benzidine-based azomethine derivatives 2, 4a-b were found to exhibit good anticancer activity, especially against MDA-MB-231.

Experimental section

Synthesis of compound 2

All commercially available chemicals were obtained from Sigma-Aldrich and used without further purification. To a 100-mL one-necked round-bottomed flask equipped with a condenser were added benzidine (1) (0.5 g, 2.71 mmol) and
ethanol (50 mL). To the above solution was added formaldehyde solution (54.28 mmol, 1.63 g, 20 equiv), and the reaction mixture was refluxed for 12 h and monitored by TLC. After complete consumption of benzidine (1), the reaction mixture was concentrated under reduced pressure. The mixture was then cooled to room temperature. Upon cooling the resulting reaction solution to ambient temperature, white solids precipitated from the solution, the solid product was filtered and washed with cold methanol and then dried in a vacuo to give the compound (2) (yield, 98%). \(N^4, N^2, N^4, N^2\)-Tetrakis(ethoxymethyl)-[1,1'-biphenyl]-4,4'-diamine (2): Mp: 137–139 °C IR (cm\(^{-1}\)): 3091, 3019, 1979, 2932, 2879, 2877, 1610, 1505, 1422, 1388, 1367, 1330, 1265, 1203, 1161, 1100, 1058, 1034, 997, 966, 897, 836, 817, 764, 751, 677, 631. \(^1^H\) NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.45 \((d, J = 8.7\) Hz, 4H), 7.11 \((d, J = 8.7\) Hz, 4H), 4.88 \((s, 8H), 3.52 \((q, J = 7.0\) Hz, 8H), 1.24 \((t, J = 7.0\) Hz, 12H). \(^1^C\) NMR (100 MHz, CDCl\(_3\)): \(\delta\) 145.60, 132.60, 127.09, 114.98, 82.76, 62.36, 15.16. HRMS (Q-TOF): m/z [(M–C\(_{10}\)H\(_{24}\)O\(_4\) + H\(^+\)] calcd. for C\(_{14}\)H\(_{13}\)N\(_2\): 209.10787, found: 209.10787.

**General procedure for the synthesis of the compounds 4a-b**

Benzidine (1) (1.000 mmol) and aromatic aldehydes 3a or 3b (2.100 mmol) were dissolved in EtOH, and the resulting reaction mixture was heated at the refluxed temperature for 12 h and monitored by TLC. After the complete consumption of aromatic aldehyde, the reaction mixture was concentrated under reduced pressure. The mixture was then cooled to room temperature. Upon cooling the resulting reaction solution to ambient temperature, crystals precipitated from the solution, the solid product was filtered and washed with cold methanol and then dried in vacuo to give the compounds (4a-b) (Mutahir et al. 2017; Göbel et al. 2003).

\(1(E, 1'E)-N, N'-((1,1'-biphenyl)-4,4'-diyl)bis(1-phenylmethanimine)\) (4a): Mp: 231–233 °C. \(^1^H\) NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.53 \((s, 2H, CH = N\), 7.98–7.89 \((m, 4H), 7.71–7.63 \((m, 4H), 7.53–7.46 \((m, 6H), 7.36–7.29 \((m, 4H)\). \(1(E, 1'E)-N, N'-((1,1'-biphenyl)-4,4'-diyl)bis(1-(4-nitrophenyl)methanimine)\) (4b): Mp: 218–220 °C. \(^1^H\) NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.62 \((s, 2H), 8.33 \((d, J = 8.8\) Hz, 6H), 8.09 \((d, J = 8.8\) Hz, 2H), 7.61 \((d, J = 8.6\) Hz, 4H), 7.33 \((d, J = 8.6\) Hz, 4H).

**Anticancer activity of the compounds 2, 4a-b**

**Cell culture**

MDA-MB-231 breast cancer and DLD1 colorectal cancer cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Diagnovum, Germany) containing 10% fetal bovine serum (FBS, Diagnovum) and 1% penicillin–streptomycin (Diagnovum). Cancer cell lines were maintained at 37 °C and 5% CO\(_2\) in an incubator. Cell lines were regularly tested for mycoplasma contamination.

**Measurement of cell survival and IC\(_{50}\)**

Cancer cells were seeded in 96-well plates at a density of 0.5×10\(^4\) in a 200 µl culture medium and incubated at 37 °C and 5% CO\(_2\) until the cells were grown to reach about 70% confluency before chemical treatments. Then, the cells were treated with different concentrations of the compound 2, 4a-b for 24 h. Cell survival was determined using the Eco-Tech CVDK-8 Cell Viability kit (EcoTech Biotechnology, Turkey) according to the manufacturer’s recommendations. The assay is based on the reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). As a result of WST-8 bio-reduction, the amount of yellow-colored formazan is produced in the culture medium and this is directly proportional to the number of living cells.

The absorbance of the medium in each well was measured at 450 nm via a spectrophotometer. Dimethyl sulfoxide (DMSO) was used as a solvent for the compounds. The same amount of DMSO (1/1000) was added to the culture medium of control groups as the amount used to dilute compounds which was not higher than the 1/1000 ratio. IC\(_{50}\) values were calculated and visualized using the online AAT Tool.

**Acridine orange (AO) and ethidium bromide (EB) dual staining**

AO can be taken in by viable cells, whereas EB only penetrates cells with compromised membrane integrity, staining their nuclei red. As a result, it enables the detection of live, dead, and apoptotic cells (Ribble et al. 2005). Cells were treated with 100 µM doses of compounds 2, 4a-b in 24-well sterile plates and incubated for 24 h. Each well was washed with PBS at room temperature and treated with AO/EB ready dye solution (including 100 µg/mL AO and EB), and cells were examined immediately with a fluorescence microscope (EVOS FL, Invitrogen).

**Statistical analysis**

The data set was created by transferring the absorbance values and folding time calculation results of the wells to IBM SPSS 20.0 software. The variance homogeneity test was applied according to the results obtained by applying the Shapiro–Wilk normality test to the created data set, and \(p < 0.05\) was considered significant. A one-way ANOVA test was applied to all the data and the appropriate post hoc tests were selected according to the variance homogeneity test results and \(p < 0.05\) was considered significant.
Instrumentation

$^1$H NMR spectra were recorded on a Bruker Ultrashield Plus Biospin spectrometer at 400 MHz. NMR chemical shifts were determined relative to internal standard TMS at $\delta$ 0.0 ppm. The chemical shifts ($\delta$) are reported in ppm, and coupling constants ($J$) are in Hertz (Hz). Melting Points ($M.p.$) were recorded on a Stuart melting point SMP30 device and are uncorrected. FT-IR spectra were recorded using a Perkin Elmer Frontier FT-IR spectrophotometer. Mass spectra were recorded on an Agilent Technologies 6530 Accurate-Mass Q-TOF-LC/MS. The absorbance of the cell culture medium was measured via an ELx800 BioTek spectrophotometer.

Molecular docking calculations

Molecular docking calculations are the common method used to compare the activities of molecules. In this method, the interactions of molecules with proteins are examined. These calculations are made using the Maestro Molecular modeling platform (version 12.2) by Schrödinger (Schrodinger 2019a). For these calculations, it is necessary to prepare both proteins and molecules, which consist of many stages. It is the preparation process of the proteins studied in the first stage. At this stage, the active sites of proteins are determined. All proteins in this active region were given freedom of movement because the molecules were easier to interact with the proteins. At this stage, the protein preparation module (Schrodinger 2019) is used. The next stage is the preparation of molecules for calculations. In this process, all conformers of the molecules are prepared. For the interaction of proteins and molecules, each structure is tested one by one, in an attempt to find the most stable interaction pose. the LigPrep module (Schroding er 2019b) is used in the calculations of this stage. In the next step, the interactions of molecules with proteins are made. At this stage, calculations are made with The Glide ligand docking module (Schrodinger 2020) for calculations. In these calculations, the preparation of molecules and proteins is calculated by the OPLS3e method. In the next stage, the properties of molecules to be drugs are examined. This analysis is performed to predict the actions of drug molecules in human metabolism. These calculations are calculated by The Qik-prop module (Tüzün 2020) of the Schrödinger software.

Results and discussion

Synthesis and characterization of the compounds 2, 4a-b

Three benzidine-based azomethine derivatives 2, 4a-b were synthesized in good yields. The synthetic routes and synthesized compounds are shown in Scheme 1. Benzidine (1) which was commercially available was used as the starting material for the preparation of target compounds 2, 4a-b. Interestingly, treatment of benzidine (1) with an excess of formaldehyde (20 equiv.) in refluxing EtOH for 12 h afforded compound 2 an excellent yield (98%). Scheme 2 shows the proposed new reaction mechanism for the synthesis of the obtained compound 2. As it is known, formaldehyde is a
very reactive type of aldehyde. It is thought that imine is formed first in the reaction medium as a result of the rapid reaction of formaldehyde with free amine groups in the benzidine skeleton. As a result of the reaction of the formed imine group with EtOH, which was used as a solvent, product 2 was obtained (Scheme 2). Synthesis of the compounds 4a-b was achieved by the condensation of benzidine (1) with 2.1 equivalents of the appropriate aromatic aldehydes 3a-b in refluxing EtOH according to a previously reported method (Iqbal et al. 2007; Mutahir et al. 2017).

The reaction progress was monitored by TLC, and when starting materials run out, the reaction was confirmed. Chemical structures of the obtained compounds were characterized by NMR, IR, and as well as HRMS spectral analyses. The molecular weights, formula, melting point (m.p), appearance and yields of the benzidine-based azomethine derivatives are shown in Table 1.

$^1$H NMR spectrum of compound 2 (Fig. 1) having a symmetrical structure consists of sets of signals appearing in aliphatic and aromatic regions. The aromatic Ha protons are split into a doublet by the ortho- or 1,2- coupling with the Hb protons 7.45 ppm ($J = 8.7$ Hz, 4Ha). The Hb protons resonate as a doublet at 7.11 ppm, due to coupling with the Ha protons ($J = 8.7$ Hz, 4Hb). The Hc protons resonate as a singlet at 4.88 ppm (8H) due to the aliphatic $-\text{CH}_2$-units. Ethoxy protons (Hd and He) resonate as a quartet at 3.52 (q, $J = 7.0$ Hz, 8Hd) and triplet at 1.24 (t, $J = 7.0$ Hz, 12He) ppm, respectively. In addition, $^{13}$C NMR spectrum of compound 2 consists of sets of signals appearing in aliphatic and aromatic regions. The proton-decoupled $^{13}$C NMR spectrum of compound 2 shows 7 carbon signals because of its symmetrical structure. The data matched very well with the structure of the molecule (Fig. 2).

The IR spectrum of compound 2 exhibited characteristic absorption bands at about 3091 cm$^{-1}$, 1388 cm$^{-1}$, 1100 cm$^{-1}$, 997 cm$^{-1}$ and 817 cm$^{-1}$. The C–H stretching band was seen at 3091 cm$^{-1}$ (Turhan et al. 2012). The asymmetric C–C stretching band was also seen at 1388 cm$^{-1}$. The

### Table 1

| Compounds | Molecular formula | Molecular weight (g/mol) | Color and appearance | m.p. °C | Yield (%) |
|-----------|------------------|--------------------------|----------------------|---------|-----------|
| 2         | C$_2$H$_{20}$N$_2$O$_4$ | 41,655                  | White powder         | 137–139 | 98        |
| 4a        | C$_{26}$H$_{20}$N$_2$ | 36,045                  | Yellow powder        | 231–233 | 95        |
| 4b        | C$_{26}$H$_{18}$N$_4$O$_4$ | 45,045                 | Brown powder         | 218–220 | 91        |
band located at 1100 cm⁻¹ was assigned to the asymmetric ring vibration (Wang and Chung 1994). Moreover, the IR spectra of compound 2 showed one characteristic band at 1330 cm⁻¹ is known as the formation of the (C─N) band and one characteristic band at 1265 cm⁻¹ is also known as the formation of the (C─O) band. The other band located at 1610 cm⁻¹ was assigned to C═C stretching band (Uddin et al. 2020; Mutahir et al. 2017) (Fig. 3).

High-resolution mass spectrometry (HRMS) confirmed the constitution of compound 2 (Fig. 4). The HRMS results demonstrated excellent agreement between the calculated mass (209.10787) and the obtained mass (209.10787) with the separation of C₁₀H₂₄O₄ atom group from the structure (m/z [(M-C₁₀H₂₄O₄) + H]+).

Anticancer activity of the compounds 2, 4a-b

After successfully synthesizing and characterizing the three benzidine-based azomethine derivatives, named compounds 2, 4a-b, we investigated whether these molecules had any anti-cancerogenic effects as anti-cancerogenic qualities of similar structures have previously been reported. We evaluated the anti-proliferative action of these compounds in breast and colon cancer cell lines at five different concentrations (1, 10, 20, 50, 100 µM) for 24 h. First, we tested 1–100 µM benzidine-based azomethine derivatives in the MDA-MB-231 breast cancer cell line. The number of breast cancers decreased compared to the control group in a dose-dependent manner in response to benzidine-based azomethine derivatives administration (Fig. 5). The compounds 2 and 4b significantly decreased the number of breast cancer cells as low as 1 µM. Among the three compounds examined, compound 2 had the highest anti-proliferative effect and killed more than 50% of the cells in response to 100 µM. The compounds 4a-b killed about 34% and 30%, respectively (Fig. 5). No cytotoxic effect was observed in DMSO added control groups. Additionally, the IC₅₀ dose of compound 2 was calculated as 123.2 µM for the MDA-MB-231 cell line (Fig. 9).

As for the DLD1 colorectal adenocarcinoma cell line, the anti-proliferative actions of these three benzidine-based azomethine derivatives 2, 4a-b were less effective for this colon cancer cell line than that of MDA-MB-231 cells. None of the compounds significantly killed the cells as low as 1–10 µM concentrations compared to the control group. The compounds 2 and 4a had similar anti-proliferative action and killed almost 38% of the cells in response to 100 µM. The compound 4b was less effective and killed only 20% of colorectal cancer cells (Fig. 6).
Fig. 2 $^{13}$C NMR (100 MHz, CDCl$_3$) spectrum of compound 2

Fig. 3 The IR spectrum of the compound 2
Among these three compounds, the most effective to kill cancerous cells was compound 2. Therefore, we calculated the IC<sub>50</sub> value of compound 2. IC<sub>50</sub> value was calculated as 121.9 µM for the DLD1 cell line (Fig. 7).

We also used a non-cancerous human umbilical vein endothelial cell line (HUVEC) to test the cytotoxicity and selectivity of compound 2. IC<sub>50</sub> doses were calculated as 123.2 µM (MDA-MB-231) and 121.9 µM (DLD1); however, these dose ranges were not significantly cytotoxic for HUVEC. The cytotoxic dose of compound 2 on HUVEC cells started at about 200 µM with less than 9% cell death (Fig. 8). These results indicate that compound 2 selectively decreases cell viability for both colon and breast cancer cells without causing cytotoxicity for non-cancerous cells.

Cancer cells (DLD1, MDA-MB-231) were treated with increasing concentrations of benzidine-based azomethine derivatives 2, 4a-b for 24 h and relative survival fraction was determined. Error bars represent one standard deviation from the mean. One-Way ANOVA: <sup>2</sup>, 4a, 4b < 0.001. PostHoc Tamhane’s T2: p<sup>ad</sup>, af, ai, ak, gi, gj, bk, bd, cm, an, ap, ar < 0.001, p<sup>ab</sup>, ac, bd, cf, dk, ao < 0.01, p<sup>ce</sup>, cf, ah, hj, mr < 0.05. PostHoc Bonferroni; p<sup>jp</sup> < 0.01, p<sup>rp</sup>; ep, bg, gm, hk, fr < 0.001. At least three independent experiments were performed.
Fig. 6  The DLD1 colorectal cancer cells were treated with 1–100 µM benzidine-based azomethine derivatives 2, 4a-b for 24 h and the relative survival fraction was determined. Error bars represent one standard deviation from the mean One-Way ANOVA; $p_{2, 4a, 4b} < 0.001$. PostHoc Tamhane’s T2: $p_{ae, af, bd, bf, cg, aj, ak, gk, ik, jk, am, an} < 0.001$, $p_{cd, ce, df, gj, no, ep} < 0.01$, $p_{bi, bo, mo, mp} < 0.05$. PostHoc Bonferroni; $p_{cn, co} < 0.05$, $p_{ej} < 0.01$, $p_{fp, kp} < 0.001$. At least three independent experiments were done.

Fig. 7  IC$_{50}$ regression results of the compound 2 for MDA-MB-231 and DLD1 cell lines. IC$_{50}$ dosages were calculated as 123.2 µM for MDA-MB-231 and 121.9 µM for DLD1 cell lines.
apoptosis (Fig. 10).

2DLD1 colorectal cancer cell line to 20 µM the compound lines at 100 µM concentration (Fig. 11).

induced apoptosis (dark and light orange color) in both cell stages were observed, while treatment of each compound very few or no apoptotic cells in either early or advanced (Fig. 11). Our results showed that in untreated, control cells EB staining assay in MDA-MB-231 and DLD1 cell lines to each of these three compounds, we performed a dual AO/

Like in MDA-MB-231 breast cancer cells, exposure of DLD1 colorectal cancer cell line to 20 µM the compound 2 caused similar morphological changes associated with the apoptosis (Fig. 10).

To test the occurrence of tumor cell apoptosis in response to each of these three compounds, we performed a dual AO/ EB staining assay in MDA-MB-231 and DLD1 cell lines (Fig. 11). Our results showed that in untreated, control cells very few or no apoptotic cells in either early or advanced stages were observed, while treatment of each compound induced apoptosis (dark and light orange color) in both cell lines at 100 µM concentration (Fig. 11).

Molecular docking calculations

It is used to compare the activities of the molecules 2, 4a-b against cancer proteins by theoretical calculations. In molecular docking calculations, which are most commonly used in these calculations, it is seen that the values closest to the results obtained as a result of experimental processes have a great harmony (Bilgiçli et al. 2021; Aktaş et al. 2021). Many parameters are calculated from molecular docking calculations. Among these calculated parameters, the most important parameter that determines the activity is the docking score. The numerical value of this parameter is expected to be negative. The more negative value the molecule is thought to have higher activity than other molecules.

The most important factor affecting the molecule to have a more negative value is the chemical interaction (Bilgiçli et al. 2021). It should be well known that the more the molecule interacts with the protein, the higher the docking score. These interactions have many interactions such as hydrogen bonds, polar and hydrophobic interactions, $\pi$-$\pi$ and halogen (Aktaş et al. 2020, 2021; Türkân et al. 2022; Gedikli et al. 2021a, b; Çetiner et al. 2021; Tüzün et al. 2021; Ataseven et al. 2021).

As a result of molecular docking calculations, in Fig. 12, it is seen that the interaction between compound 2 and breast cancer protein, and in compound 2, bis(ethoxymethyl)amine group makes hydrogen bonds with ARG 1699 protein. Besides, in the interaction between compound 2 and colon cancer protein in Fig. 13, hydrophobic and unspecified residue interactions occurred between compound 2 and colon cancer protein. In Fig. 14, in the interaction between compound 4a and breast cancer protein, it was seen that there was a Pi-cation interaction with the benzene ring ARG1835 protein in the center of compound 4a. It is observed that hydrogen bonding occurs between the $N$ atom in one of the phenylmethanimine groups on both sides of compound 4a and the LEU 1701 protein and a Pi-cation interaction occurs between ARG 1699 and the benzene ring in the phenylmethanimine group on the other side. Figure 15 shows that in the interaction between compound 4a and colon cancer protein, hydrophobic interactions occur between compound 4a and colon cancer protein. In Fig. 16, in the interaction between compound 4b and breast cancer protein, it is seen that pi-cation interaction occurred between the benzene ring in the center of compound 4b and the ARG 1835 protein. On the other hand, it is seen that the nitro groups at the ends of the molecule occur as a pure bridge interaction with the GLU 1698 and ASP 1813 proteins, respectively. Finally, in Fig. 17, in the interaction between compound 4b and colon cancer protein, it is seen that hydrophobic interaction occurs between compound 4b and colon cancer protein.

In comparing the activities of molecules 2, 4a-b, it is not sufficient to examine the docking score parameter only. It is necessary to examine other calculated parameters. All parameters obtained from molecular docking calculations are visualized in Table 2. Some of these other parameters are Glide hbond, Glide evdw, and Glide ecoul parameters. These parameters are the chemical interactions of molecules with cancer proteins (Aktaş et al. 2020). The interaction of molecule 2 with the highest activity is given in Figs. 12–13. Other remaining parameters are Glide emodel, Glide energy, Glide einternal, and Glide posenum parameters. These parameters provide numerical information for the interaction of molecules with cancer proteins (Gedikli et al. 2021a; Çetiner et al. 2021).

A comparison of the activities of the molecules 2, 4a-b against cancer proteins was made. It is not enough for
a molecule to be a drug only if its activity is high. How these molecules act in human metabolism is very important to become drugs. Because when molecules enter human metabolism and cause toxic effects, they can cause human death (Gedikli et al. 2021b). Therefore, ADME/T analysis of molecules is performed. With this analysis, the route of entry of molecules in human metabolism, their interaction, excretion by human metabolism, and the toxic effects they cause have been examined in detail (Tüzün et al. 2021; Ataseven et al. 2021). All calculated ADME/T parameters are given in Table 3.

As a result of this investigation, many parameters were found. One of these parameters found is the molar mass (mol_MW). Because it is desired that the mole cult is neither too little nor too much (Aktaş et al. 2021). On the other hand, it is desired that the dipole moment (dipole (D)) of the molecules should be neither too little nor too much (Aktaş et al. 2020). Another parameter is the number of hydrogen bonds (donorHB and acceptHB) taken or given between molecules and proteins. There are many parameters such as the numerical information of the passage of molecules through the blood intestine (QPPCaco) and blood–brain (QPPMDCK) barriers (Gedikli et al. 2021a; Çetiner et al. 2021). It is seen that compounds 2 and 4a are not suitable for these parameters. Parameters such as RuleOfFive and RuleOfThree are the parameters that decide whether the molecules are suitable for use as drugs because these parameters are made up of many parameters (Gedikli et al. 2021b; Tüzün et al. 2021; Ataseven et al. 2021). Therefore, the numerical value of the RuleOfFive and RuleOfThree parameters is expected to be zero. When the parameters contained in these parameters do not meet the required conditions, the number increases for each parameter. Therefore, it is considered appropriate to be used as a medicine if all the parameters they contain provide the necessary conditions.

**Conclusions**

A benzidine-based azomethine derivative 2 having a proposed new mechanism and its other two derivatives 4a-b have been successfully synthesized in high yields by reaction of benzidine (1) with formaldehyde, benzaldehyde 3a or nitrobenzaldehyde 3b. The structural characterization of the compounds 2, 4a-b has been evaluated by using NMR, FT-IR, and HRMS. Ultimately, the synthesized compounds

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Fig. 9 Light microscopy images of the live breast cancer cells (MDA-MB-231) that were treated with benzidine-based azomethine derivatives 2, 4a-b for 24 h. At least three independent experiments were performed, and representative images are shown. Scale bar: 400 µm
were evaluated for their anticancer activities against human breast cancer cells (MDA-MB-231) and human colon cancer cells (DLD1). They have shown anticancer potential against these cancer cells. The results showed that the benzidine-based azomethine derivatives 2, 4a-b had a significant effect on the human breast cancer

Fig. 10 Light microscopy images of the live colon cancer cells (DLD1) that were treated with benzidine-based azomethine derivatives 2, 4a-b for 24 h. At least three independent experiments were performed, and representative images are shown. Scale bar: 400 µm

Fig. 11 AO (green) and EB (red) fluorescent staining of MDA-MB-231 and DLD1 cell lines treated with 100 µM doses of compounds 2, 4a, and 4b (Scale bars 200 µm). Uncutted arrows with a head indicate live cells; cut arrows with a head indicate apoptotic cells; cut arrows with no head indicate dead cells
cell line (MDA-MB-231). Compound 2 had the highest anti-proliferative effect against (MDA-MB-231) and killed more than 50% of the cells in response to 100 µM. The compounds 4a-b killed about 34% and 30%, respectively. The results of this research encourage us to develop other similar related compounds and test them for a wide range of anticancer activity. However, it is seen that there is a great agreement between the theoretical studies and the experimental results. According to the results of molecular docking, it is seen that it is compound 2 with −3.43 against breast cancer protein and −4.91 against colon cancer protein. According to the ADME/T results performed afterward, although the molecules 2, 4a-b have problems in some parameters, it is thought that there will be no problem in the use of more detailed in vivo and in vitro experiments to be carried out in the future. Although theoretical calculations will guide future studies, it is impossible to predict what effects the molecules will have on different human metabolism.
Fig. 14  Presentation interactions of compound 4a with breast cancer

Fig. 15  Presentation interactions of the compound 4a with colon cancer
Fig. 16  Presentation interactions of compound 4b with breast cancer

Fig. 17  Presentation interactions of the compound 4b with colon cancer
Table 2 Numerical values of the docking parameters of molecules 2, 4a-b against proteins

| Compound | 2 | 4a | 4b |
|----------|---|----|----|
| **Breast cancer** | | | |
| Docking score | −3.43 | −3.37 | −3.27 |
| Glide ligand efficiency | −0.10 | −0.12 | −0.10 |
| Glide hbond | −0.16 | −0.21 | −0.13 |
| Glide evdw | −31.02 | −30.51 | −34.22 |
| Glide ecol | −6.64 | −2.04 | −2.03 |
| Glide emodel | −43.95 | −37.83 | −41.83 |
| Glide energy | −37.67 | −32.55 | −36.25 |
| Glide einternal | 2.67 | 4.14 | 2.91 |
| Glide posenum | 90 | 43 | 383 |
| **Colon cancer** | | | |
| Docking score | −4.91 | −4.83 | −4.40 |
| Glide ligand efficiency | −0.14 | −0.17 | −0.13 |
| Glide hbond | −0.24 | 0.00 | 0.00 |
| Glide evdw | −37.58 | −34.33 | −39.59 |
| Glide ecol | −6.48 | 0.56 | −0.37 |
| Glide emodel | −51.87 | −42.35 | −49.05 |
| Glide energy | −44.06 | −33.76 | −39.96 |
| Glide einternal | 12.74 | 5.21 | 5.55 |
| Glide posenum | 95 | 307 | 132 |

Table 3 ADME properties of the molecules 2, 4a-b

| 2 | 4a | 4b | Reference range |
|---|----|----|----------------|
| mol_MW | 417 | 360 | 450 | 130–725 |
| Dipole (D) | 2.5 | 3.2 | 6.7 | 1.0–12.5 |
| SASA | 867 | 727 | 806 | 300–1000 |
| FOSA | 620 | 45 | 45 | 0–750 |
| FISA | 8 | 30 | 213 | 7–330 |
| PISA | 239 | 652 | 528 | 0–450 |
| WPSA | 0 | 0 | 0 | 0–175 |
| Volume (Å³) | 1525 | 1279 | 1429 | 500–2000 |
| donorHB | 0 | 0 | 0 | 0–6 |
| accepHB | 8.8 | 2 | 4 | 2.0–20.0 |
| Glob (Sphere = 1) | 0.7 | 0.8 | 0.8 | 0.75–0.95 |
| QPpolrz (Å³) | 47.5 | 46.7 | 50.3 | 13.0–70.0 |
| QPlogPC16 | 14.6 | 15.0 | 17.1 | 4.0–18.0 |
| QPlogPtot | 19.4 | 16.7 | 20.0 | 8.0–35.0 |
| QPlogPW | 8.8 | 6.5 | 8.8 | 4.0–45.0 |
| QPlogPo/w | 5.2 | 7.2 | 5.7 | 2.0–6.5 |
| QPlogS | −5.6 | −7.8 | −7.9 | −6.5–0.5 |
| CIQPlogS | −4.6 | −7.2 | −8.2 | −6.5–0.5 |
| QPlogHERG | −6.9 | −8.1 | −7.9 | (Concern below −5) |
| QPPCaco (nm/sec) | 8278 | 5144 | 61 | * |
| QPlogBB | −0.4 | −0.2 | −2.7 | −3.0–1.2 |
| QPPMDCK (nm/sec) | 4859 | 2905 | 24 | * |
| QPlogKp | 0.6 | 0.9 | −3.1 | Kp in cm/hr |
| IP (ev) | 9.5 | 8.6 | 9.1 | 7.9–10.5 |
| EA (eV) | 0.1 | 0.9 | 2.5 | −0.9–1.7 |
| #metab | 6 | 0 | 2 | 1–8 |
| QPlogKhsa | 0.1 | 1.5 | 1.3 | −1.5–1.5 |
| Human oral absorption | 3 | 1 | 1 | – |
| Percent human oral absorption | 100 | 100 | 79 | ** |
| PSA | 42 | 22 | 116 | 7–200 |
| RuleOfFive | 1 | 1 | 1 | Maximum is 4 |
| RuleOfThree | 0 | 1 | 1 | Maximum is 3 |
| Jm | 4.3 | 0.0 | 0.0 | – |

* < 25 is poor and > 500 is great. ** < 25% is poor and > 80% is high

Stars: Number of property or descriptor values that fall outside the 95% range of similar values for known drugs; Amine: Number of non-conjugated amine groups; rtvFG: Number of reactive functional groups; SASA: Total solvent accessible surface area; FOSA: Hydrophobic component of the SASA; FISA: Hydrophilic component of the SASA; PISA: π (carbon and attached hydrogen) component of the SASA; WPSA: Weakly polar component of the SASA; donorHB: Estimated number of hydrogen bonds that would be donated; AccepHB: Estimated number of hydrogen bonds that would be accepted; QPpolrz: Predicted polarizability in cubic angstroms; QPPCaco: Predicted apparent Caco-2 cell permeability in nm/sec; QPlogBB: Predicted brain/blood partition coefficient; QPPMDCK: Predicted apparent MDCK cell permeability in nm/sec; QPlogKp: Predicted skin permeability; meta: Number of likely metabolic reactions; QPlogKhsa: Prediction of binding to human serum albumin; PercentHuman-OralAbsorption: Predicted human oral absorption on 0 to 100% scale; PSA: Van der Waals surface area of polar nitrogen and oxygen atoms; RuleOfFive: Number of violations of Lipinski’s rule of five; RuleOfThree: Number of violations of Jorgensen’s rule of three.

Funding This research has been supported by Kafkas University Scientific Research Projects Coordination Unit. Project Number 2021-FM-66 Year 2021.

Declarations

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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