Inhibition of T24 and RT4 Human Bladder Cancer Cell Lines by Heterocyclic Molecules

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Background: Bladder cancer is a major widespread tumor of the genitourinary tract. Around 30% of patients with superficial cancers develop invasive and metastatic pathology.

Material/Methods: Some new heterocyclic 4-methyl coumarin derivatives were designed using molecular modeling studies to evaluate their potential against bladder cancer lines T24 and RT-4. The designed compounds that showed good binding affinity to T24 and RT4 were synthesized, with excellent yield. The synthesized compounds after structural evaluation were further evaluated for their antiproliferative activity by cell viability assay, cell cycle analysis, and apoptosis assay.

Results: The compound BC-14 exhibited the best cytotoxicity against T24 cells, but were not highly active against RT4 cells.

Conclusions: The results of the present study may suggest the selectivity pattern of the synthesized compounds. These results should be explored further with chemical modification for other cancer types.

MeSH Keywords: Cell Cycle • Drug Design • Urinary Bladder Neoplasms

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Background

Bladder cancer begins when cells in the urinary bladder start to grow uncontrollably. As more cancer cells develop, they can form a tumor and spread to other areas of the body. They arise from the epithelial lining (the urothelium) of the urinary bladder. Rarely, the bladder is the site of non-epithelial cancers, such as lymphoma or sarcoma, but these are not ordinarily included in the colloquial term “bladder cancer.” It is a disease in which cells abnormally multiply out of control in the bladder. The intravesical administration of Bacillus Calmette-Guerin (BCG) after transurethral resection is the most efficient action of superficial bladder cancer, but adverse effects of BCG therapy are common, and it has been reported that one-third of patients do not respond to this treatment. As various treatments may be ineffective, the search for more effective and selective target compounds for treatment of bladder cancer is essential [1–3].

Components of the plasminogen activator system provide important mechanisms for tumor implantation. The plasminogen activator system is involved in bladder cancer. These levels are increased in associated with urothelial cancer. The u-PA receptor (u-PAR) is hypothesized to focus and prolong effective plasminogen activation, enhancing basement membrane destruction and tumor invasion while preventing inactivation by inhibitors. Determination of the u-PAR in the human transitional cell carcinoma cell lines RT4 and T24 co-relate its involvement in the progression and severity of the bladder cancer [3].

The discovery that warfarin is effective against the V2 cell lines of granulocytes, macrophages, lymphocytes, and Umbelliferone, which is use in lung cancer treatment, has attracted the attention of researchers because coumarin is the pharmacophore in both of these drugs. Coumarin is considered to be a versatile nucleus upon which various pharmacological activities (e.g., anticancer [4–10], antibacterial, antiviral, and antioxidant) are reported. Coumarin and its derivatives are reported to produce significant changes in the immune response to cell growth and proliferation. They are also involved in inhibition of protein kinase and regulation of expression by the oncogens [4–10].

Some research reports revealed that natural coumarins exhibit cytotoxic potential against glioma cells, with IC_{50} values ranging from 31 to 34, and their effectiveness against organotypic cell cultures shows their selectivity against these cancer cell lines. These reports encouraged us to synthesize some new heterocyclic coumarin derivatives and evaluate the potential of the designed molecules against bladder cancer cell lines by using drug design concepts. Moreover, our literature search found that 4-methyl coumarin is reported to have a prominent cytotoxicity profile against bladder cancer cell lines.

The presence of a methyl group at the 4th position protects the scaffolds from metabolism by the liver cytochrome P450 enzymes to mutagenic derivatives. Moreover, these derivatives lack the anticoagulant effect of warfarin derivatives [12–16].

Our literature search showed that some new heterocyclic 4-methyl coumarins were designed and subjected to molecule docking to study the inhibition of the T24 and RT4 [17–25].

Material and Methods

Molecular docking

Ligand preparation

The structure of 3-7/8-disubstituted-4-methyl coumarin derivatives was used as the pharmacophore to design a new set of molecules in the V-Life Molecular Design Suite 3.5. Optimizations of the ligands were done by using an MMFF force field until a gradient of 0.001 kcal/mol/Å was reached. The template structure was set to be rigid while generating other datasets. The ligand structure was imported to the new window with proper names and the program was run to prepare the ligand. Finally, the input ligand was selected (molecules: all) on a protein molecular window.

Preparation of the grid file

The active pockets of the protein were centered with the grid map by V-Life MDS 3.5 [22] (Figure 1). The grid map was centered by the amino acid residues.

Preparation of the docking file

We obtained the structure of the biological target 2-BAX from the Protein Data Bank (www.rcsb.org/pdb) and set it as a target. Optimization of the protein structure was done by using energy minimization method. MMFF was the force field used for optimization with the help of VLife MDS 3.5 software up to the RMS gradient of 0.01. The generated conformers of the designed molecules were also subjected to energy minimization by using the same procedure. The selection of active site was based on maximum hydrophobic surface. The GA docking method was used for docking simulation. All the conformers were then docked at the active site of the protein. For rotation angle, the number of placements was set at the value of 30 to check the flexibility of these conformers at different rotational angles because different rotations of the dock score have different values. Each value was then saved to find the best fit. In docking results, only the best fit results were had good dock scores. Different interactions were noted and analyzed. The docking interactions are shown in Figure 2–4.
Synthetic studies

General

All chemicals used for synthesis were of laboratory grade. The NMR spectra was taken by using a Bruker AC-400 NMR spectrometer and TMS as an internal standard. FTIR spectra were recorded using a Schmadzu FTIR spectrophotometer. The melt-ing point was assessed by open capillary method and were uncorrected. TLC analysis was performed on pre-coated silica plates (Merck).

Synthetic procedure for BC-2 and BC-3

A sulfuric acid mixture of resorcinol (1 mmol) in Substituted ethyl acetoacetate (15.23 mmol) was added slowly at 0°C. The resultant mixture was stirred at room temperature. The mixture obtained was poured onto crushed ice. Crude solid was filtered, washed with water, and dried. Rec. Spirit was used for recrystallization.

BC-2: Pale yellow solid (1.78 g; 78%) mp: 111–113°C. 1H NMR (methanol, 400 MHz): d 0.84 (t, 3H, J=6.9 Hz, –Hexyl), 1.17–1.38 (m, 8H, –Hexyl), 2.29 (s, 3H, C-4 Me), 6.61 (d, 1H, J=1.9 Hz, 8H), 6.34(dd, 1H, J=1.99 and 7.9 Hz, 6H), 7.31 (d, 1H, J=7.9 Hz, C–6H);13C NMR (CH3OH-d4, 100 MHz): 12.82 (–Hexyl), 13.01(C-4, Me), 21.50, 24.31, 23.81, 28.41, 30.21 (–(CH2)5CH3), 100.67(C-8),110.65, 110.01, 116.64 and 119.66 (C-3, C-5, C-6 and C-10),137.46 (C-4), 151.49 and 159.29 (C-7 and C-9), 151.86 (C-2, C=O);IR (Nujol) mmax: 3411.2 (OH), 1711.3 (CO), 1359.9, 1220.7, 490.0 cm⁻¹; EI MS: C16H20O3 [M]+: 260.20.

BC-3: White Solid (2.0 g, 76%); mp: 57–59°C; 1H NMR (CCl4-d, 400 MHz): d 0.80 (t, 3H, –Decyl), 1.11–1.39 (m, 16H, –Decyl), 2.29 (s, 3H, C-4 Me), 2.49 (t, 2H, J = 7.6 Hz, –Decyl), 6.54 (d, 1H, J=7.9 Hz, C–6H); 13C NMR (CCl4-d, 100 MHz): d 14.19 (–(Me)9Me),14.78 (C-4 Me), 21.68, 24.08, 25.63, 28.33, 30.01, 29.43, 31.89, 42.88 (–(Me)9Me), 103.04 (C-8), 112.45, 112.99, 122.02, IR (Nujol) mmax: 3420.1 (OH), 1717.8 (CO), 1670.5, 1610.3, 1580.1, 1507.3, 1450.6,1375.2, 1351.4, 1306.3, 1242.4, 1212.7, 1128.8, 1124.2, 1093.2, cm⁻¹; EI MS: C16H20O3 [M]+: 317.2120, found 317.2121.
**Procedure for synthesis of BC-8, BC-10, and BC-11**

In sulfuric acid, a mixture of pyrogallol (7 mmol) in Substituted C2H5COOCH3 (10 mmol) was added slowly at 0°C. The resultant mixture is stirred at room temperature. The mixture obtained was poured into crushed ice. Crude solid was filtered with water and dried. Rec. Spirit was used for recrystallization.

**BC-8**: White color solid, (1.23 g, 72%); mp: 215–217°C; 1H NMR (acetone-d6, 400 MHz): d 1.01 (t, 3H, J=6.9 Hz, –Ethyl), 2.15 (s, 3H, C-4 Me), 2.38 (q, 2H, J=6.9 Hz, –Ethyl), 6.41 (d, 1H, J=8.5 Hz, 6H), 6.99 (d, 1H, J=8.5 Hz, C-5H); 13C NMR (CH3COCH3-100 MHz): d 11.83 (–Ethyl), 12.86 (C-4 Me), 21.35 (–Ethyl), 110.11, 111.35, 113.01 and 122.35 (C-3, C-5, C-6 and C-10), 130.56 (C-8), 140.65 and 147.75 (C-7 and C-9), 150.76 (C-4), 159.57 (C-2, C=O); IR (Nujol) mmax: 3323.8 (OH), 1674.7 (C=O), 1606.6, 1504.6, 1461.3, 1372.8, 1325.4, 1248.8, 1286.5, 1136.8, 1146.8, 1157.0, 1099.0, 1119.9, 965.6, 860.0, 801.3, 769.1, 721.8 cm⁻1; M+2=219.

**BC-10**: Colorless solid, (1.60 g, 73%); mp: 120–121°C; 1H NMR (methanol-d4, 400 MHz): d 0.85 (t, 3H, –Hexyl), 1.15–1.30 (m, 8H, –Hexyl), 1.54–1.75 (m, 1H, J=7.4 Hz, –Hexyl), 6.60 (d, 1H, J=8.4 Hz, C–6H), 6.98 (d, 1H, J=7.9 Hz, C–5H); 13C NMR (CH3OH-d4, 100 MHz): d 12.54 (–Hexyl), 13.57 (C-4, Me), 22.44, 21.66, 25.81, 28.90, 30.43 (–Hexyl), and 147.14 (C-7 and C-9), 148.33 (C-4), 161.52 (C-2 carbonyl); IR (Nujol) mmax 3346.3 (OH), 1717.5 (CO), 1607.1, 1081.1 cm⁻1; M+2=276.20.

**BC-11**: Colorless solid, (1.85 g, 74%); mp: 117–119°C; 1H NMR (methanol-d4, 400 MHz): d 0.80 (t, 3H, –Decyl), 1.19–1.23 (m, 16H, –Decyl), 2.30 (3H, C-4 Me), 2.51 (t, 2H, J=7.8 Hz, –Decyl), 6.66 (d, 1H, J=7.9 Hz, C–6H), 6.11 (d, 1H, J=7.9 Hz, C–5H); 13C NMR (CH3OH-d4, 100 MHz): d 12.78 (–Decyl), 13.85 (C-4 Me), 24.01, 25.90, 27.91, 29.03, 29.33, 28.29, 29.54, 29.59, 30.65 (–Decyl), 110.91, 114.21, 112.98, and 120.91, 132.03 (C-8), 136.25 and 140.69 (C-7 and C-9), 147.03 (C-4), 161.53 (C-2 carbonyl); IR (Nujol) mmax 3329.7 cm⁻1 (OH), 1674.7 (C=O), 1606.6, 1505.5, 1463.2, 1368.7, 1334.3, 1257.4, 1293.7, 1170.0, 1135.8, 1113.8, 1102.1, 964.5, cm⁻1; M+2 =332.23.

**Procedure for synthesis of BC-14**

Mixture of compound BC-11 (400 mg), DAP (10 mg) in THF was prepared. To this mixture CH3OH was added. The mixture is stirred at room temperature for 24 h. In the resultant mixture ice-cold water was added. The solid products precipitated out was filtered with water and recrystallized from rec. spirit.

**BC-14**: Colorless solid, (0.40 g, 69%); MP: 91–93°C; 1H NMR (CDCl3-d, 300 MHz): d 0.82 (t, 3H, J=1.9 Hz), 1.19–1.19 (m, 16H, –C10H21), 2.30 (3H, C-4 Me), 2.39 (6H, 2 (–Acetoxy), 2.58 (t, 2H, J=7.6 Hz, –C10H21), 7.10 (d, 1H, J=7.9 Hz, C-6H), 7.67 (d, 1H, J=9.1 Hz, C–6H); 13C NMR (CDCl3-d, 75.5 MHz):

d 12.55 (–C10H21), 13.53 (C-4 Me), 18.95 and 19.17 (2–Acetoxy), 21.54, 26.12, 88.11, 90.05, 30.32, 38.07, 38.26, 38.64, 38.91 (–CH2)9CH3), 116.44, 120.13, 118.55 IR (Nujol) mmax: 1765.3 (CO), 1710.4 (CO), 1610.0, 1552.1, [M]+414.67.

**Anticancer studies**

**Cell lines and cell treatment**

T24 and RT4 cell line were obtained from (ATCC, Rockville, MA). RPMI culture medium was used for maintenance of T24 cells and DMEM culture medium was used for RT4 cell lines. Relative humidity was maintained at 95%.

The synthesized compounds were dissolved in DMSO and a final concentration is 0.25% (v/v) DMSO was used as a vehicle group for the experiment. This concentration has no effect on the on the prepared cell culture and does not interfere in the experiment results. The T24 and RT4 cell lines were seeded as detailed above and the cell culture was exposed to the synthesized compounds at the concentration of 5, 10, 25, 50, and 100 µM for 24 or 48 h.

**Cell viability assay**

Cell viability assay was performed according to the standard protocol prescribed under Tetrazolium Reduction Assays [3]. We used 96-well plates for the experiment. DMSO treated cells were used as a vehicle group. The cell viability assay was performed for 24–48 h. The results mentioned are given as percent in co-relation with the vehicle group.

**Cell counting**

T24 cells were prepared according to the procedure mentioned above. After 24 h of treatment, Ca and Mg buffer with EDTA solution was used for washing of cultured cells and cells were counted using a hemocytometer. The results are given as percent in co-relation with the vehicle group.

**Cell cycle analysis**

The T24 cells were plated in 6-well plates at 3.5×10³ cells/well and incubated for 72 h to attain adherence. The cells were then rated using compound BC-14 at the concentration of 25, 50, and 100 µM for 24 h. The cells were then centrifuged for 6 min, suspended in PBS, and counted. Flow cytometry was used for data collection. FLOWJO® software was used for analysis of data.

**Flow cytometric staining technique (Annexin V/PI)**

Apoptotic cells were double-stained using an annexin V–FITC–propidium iodide (PI) kit and the staining was performed as detailed above and the cell culture was exposed to the synthesized compounds at the concentration of 5, 10, 25, 50, and 100 µM for 24 or 48 h.

**Indexing:**

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according to the kit instructions. FLOWJO® software was used for analysis of data.

**Statistical analysis**

The experiments were performed in triplicate and means are reported. One-way ANOVA was used to check the statistical significance of the experiment. The distinction was considered significant with respect to the vehicle group at p<0.05.

**Results**

**Docking protocol**

Molecular docking studies: A molecular docking study was performed according to the protocol for the V-life Sci MDS model. The structures of 3–7/8 substituted coumarin derivatives were used as ligands and were docked to BAX protein (Figure 1). The results of docking are shown in Table 1 and Figures 2–4.

**Synthetic studies**

Based on the result ofdockings, the compounds showing good binding affinity with the receptor were further selected for the synthetic studies.

The designed derivatives were synthesized using Pechman condensation of phenols with alkylated ethyl acetoacetate [23–28]. The alkylation of ethyl acetoacetate using alkyl halide gives 2-alkyl ethyl acetoacetate derivatives [29,30]. The compounds BC-2 and BC-3 were synthesized by the reactions of resorcinol with alkyl-substituted ethyl acetoacetate. Similarly, compounds BC-8, BC-10 and BC-11 were synthesized by the reactions of pyrogallol with ethyl acetoacetate in acidic conditions [31–35]. The compound BC-14 was synthesized using BC-8, BC-10, and BC-11 with acetic anhydride. The structures of all the synthesized compounds in this study were confirmed by their physical constants and spectroscopic studies and were compared with results reported in the literature.

**Anticancer activity**

The inhibitory potential of the designed drug on T24 and RT4 bladder cancer lines was evaluated using the MTT assay method. Among all the compounds tested, compound BC-14 exhibited the best cytotoxic effect against T24 cell lines. As shown in Figure 5, among all the compounds tested, compound BC-14 exhibited the best cytotoxic profile and significantly reduced cell viability at the concentration of 25 µM (17.17%) after 48 h of treatment (Figure 6). Because the concentrations of 50 µM (46.11%) and 100 µM (62.16%) showed clear reductions in cell viability (Figure 6).

These compounds were tested for activity against RT4 human bladder cancer cell lines, but none of the compounds were found to be active (Figure 7A, 7B). RT4 is representative of non-invasive superficial cancer and T24 is representative of invasive bladder tumor with metastatic profile. We found that the compounds showing better activity in cell viability in T24 cell lines are not as active in MTT assay of RT4 cell lines.

To understand the mechanism involved in decreased T24 cell viability in association with cell progression, a flow cytometry study was performed on cells treated with 25–100-µM concentrations for 24 h. The results of cell cycle analysis showed that large cell accumulation in G1 phase at the concentrations of 10 and 25 µM. Because the results showed the accumulation of cells in G1 phase, the induction of apoptosis was analyzed by flow cytometry using annexin-stained cell lines. The results of cell cycle analysis showed that large cell accumulation in G1 phase (Figure 8) at the concentrations of 10 and 25 µM. Because the results showed the accumulation of cells in G1 phase, the induction of apoptosis was analyzed by flow cytometry using annexin-stained cell lines. The results in Figure 9 show an increase in apoptosis by compound BC-14 at the 100-µM.

**Discussion**

It was clear from the docking studies that the 4-methyl coumarin moiety is surrounded by hydrophobic regions. The docking models reveal that N-1 of the coumarin forms hydrogen bonds with amino acid. The compound forms hydrogen bonds with hydrogen atoms of the amino acid backbone. Ligands with

![Figure 5. Effect of designed compounds on cell viability in T-24 cell lines of human bladder cancer cell lines. DMSO is the vehicle group and cell were grown on 86-well plate. After one day the cell viability was assayed at the concentration of 5, 10, 25, 50 and 100 µM. The experiment performed in triplicate and mean is reported. One way ANOVA study was used.](image-url)
Table 1. Docking analysis.

| Compound | Substitutions | Hydrogen Bond Distance between 4-methyl coumarin derivative and amino acid backbone | Hydrophobic interaction | PLP dock score (kcal/mol) | Free energy | RMSD |
|----------|---------------|-----------------------------------------------------------------------------------|------------------------|--------------------------|-------------|------|
| BC-1     | R1: C2H5; R2: OH; R3: H | 1.87 | VAL10A, GLY11A, ILE14A | -52.22 | -14.11 | 8.16 |
| BC-2     | R1: C6H5; R2: OH; R3: H | 1.67 | VAL10A, GLY11A, SER15A | -63.53 | -15.13 | 3.26 |
| BC-3     | R1: C1OH21; R2: OH; R3: H | 1.65 | VAL10A, GLY11A, SER15A | -65.56 | -14.46 | 4.25 |
| BC-4     | R1: CH2COOEt; R2: OH; R3: H | 2.32 | VAL10A, GLY11A, SER15A | -23.18 | -11.13 | 6.77 |
| BC-5     | R1: C2H4COOEt; R2: OH; R3: H | 3.67 | VAL10A, GLY11A | -31.42 | -8.11 | 9.11 |
| BC-6     | R1: CH2COOEt; R2: OAc; R3: H | 2.11 | VAL10A, GLY11A, SER15A-45.17 | -29.13 | -9.27 | 6.27 |
| BC-7     | R1: C2H4COOEt; R2: OAc; R3: H | 2.16 | LEU33A, GLU34A, GLY11A, SER15A | -37.47 | -8.11 | 7.13 |
| BC-8     | R1: C6H5; R2: OH; R3: OH | 2.01 | LEU33A, GLU34A, GLY11A, SER15A | -59.23 | -15.01 | 4.24 |
| BC-9     | R1: H; R2: OH; R3: OH | 2.34 | LEU33A, GLY11A, SER15A | -51.23 | -14.21 | 5.66 |
| BC-10    | R1: C2H5; R2: OH; R3: OH | 2.11 | VAL10A, GLY11A, SER15A | -69.13 | -15.22 | 4.23 |
| BC-11    | R1: C1OH21; R2: OH; R3: OH | 2.11 | LEU33A, GLY11A, SER15A | -67.17 | -14.56 | 5.11 |
| BC-12    | R1: CH2COOEt; R2: OH; R3: OH | 3.12 | ALA35A, GLY11A, SER15A | -38.17 | -9.10 | 8.19 |
| BC-13    | R1: C2H4COOEt; R2: OH; R3: OH | 2.74 | ALA35A, GLY11A, SER15A | -43.27 | -8.49 | 7.23 |
| BC-14    | R1: H; R2: OAc; R3: OAc | 2.21 | VAL10A, GLY11A, SER15A | -68.87 | -14.17 | 3.19 |
| BC-15    | R1: CH2COOEt; R2: OAc; R3: OAc | 3.11 | VAL10A, GLY11A, SER15A | -39.45 | -12.43 | 7.77 |
| BC-16    | R1: C2H4COOEt; R2: OAc; R3: OAc | 3.26 | ARG42A, GLY11A, SER15A | -31.12 | -13.47 | 6.78 |
| BC-17    | R1: CH2COOEt; R2: OCH3; R3: OCH3 | 3.47 | VAL10A, GLY11A | -44.23 | -12.11 | 9.23 |
| Lead     | Erlotinib | 1.21 | VAL10A, GLY11A, SER15A, ARG42A | -89.31 | -19.49 | 0.46 |

The Bold compounds which show good dock score are taken for synthetic studies.
Figure 6. Cell viability in T24 bladder cancer cell lines by BC-14 on 96 well plate. The concentration use is 5, 10, 15, 25, 50 and 100 µM. The experiment performed in triplicate and mean is reported. One way ANOVA study was used for statistical analysis.

Figure 7. (A) Cell viability in RT4 bladder cancer cell lines in concentration of 5, 10, 25, 50, 100 µM. (B) Cell count assay in T24 human baldder cancer cell lines in concentration on of 10, 25, 50, 100 µM. The cell were grown on 96 plate wall. All the experiments are performed in triplicate and mean are reported. One way ANOVA study was used for statistical analysis.

Figure 8. Effect compound BC-14 on% cell cycle phase at the concentration 10, 25, 50, 100 µM. DMSO trated T24 cell lines are considered as vehicle group. The study performed for the period of 24 h by flow cytometry. The values for cell distribution in G1, S and G2/M phase are relative number. The data obtained by individual experiment.

Figure 9. Results of early apoptotic effect of compound BC-14. DMSO trated T24 cell lines are considered as a vehicle group. The concentration use for the compound BC-14 is 25, 50 and 100 µM. The time period for the study is 24 h. The cells were stained with annexin. The experiment performed in triplicate and mean is reported.

good docking scores form a stable complex with the receptor. The compounds BC-2, BC-3, BC-8, BC-10, BC-11, and BC-14 had good docking scores (59.23–69.13), with minimum RMSD values (3.26–5.11). The C-3 alkyl moiety showed good docking in-teractions. Other compounds showed low-to-moderate docking scores compared to active derivatives, showing that the presence of an ester linkage is not necessary for the activity.
The compound BC-14 demonstrated a good cell viability profile. Comparative data of the compounds BC-2, BC-3, BC-8, and BC-10 with BC-14 show that the presence of the carbonyl group may increase the cytotoxicity profile for these drugs.

The Result of RT4 inhibition shows that, none of the compound is active. T24 has much faster metabolism and divides more readily than RT4 cell lines, which might be why the drug metabolism is accelerated. The adverse effect of the anticancer drugs is due to the accelerated metabolism and high levels of cell proliferation, similar to that of skin and hair. We also found that there are genetic differences between RT4 and T24 cell lines. This could be another reason why compound BC-14 is less active against RT4 cell lines. Cell cycle analysis shows large cell accumulation in G1 phase which might be an effect of the characteristic apoptotic death.

Conclusions

The designed compounds with good docking score were synthesized. The synthesized compounds after structural conformation were assessed for their activity against bladder cancer cell lines. The compounds exhibited good-to-moderate activity against T24 cells. Compound BC-14 significantly reduced the viability of T24 bladder cancer cell lines, but were not as active against RT4 cells. This may show the selectivity pattern of the synthesized compounds. SAR studies of the compound reveals that C3 alkyl moiety is necessary for the cytotoxic activity. Replacement of ethyl with higher analogs may be beneficial. This might be because of the increase in the lipophilicity of the compounds. The presence of a hydroxyl group, as in BC-8, BC-10, and BC-11, also imparts significant cytotoxic activity, perhaps because these hydroxyl groups participated in the hydrogen bond formation with the amino acid. The fact that the compound BC-14 was the most active suggests that the presence of an Acetoxyl group at C7 and C8 significantly increases the activity. In addition, compound BC-14 increased the number of cells in G1 phase and induced apoptosis, suggesting the need for further exploration of this compound using an in vivo bladder cancer animal model.

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