Synthesis and Biological Activities of Some New Benzotriazinone Derivatives Based on Molecular Docking; Promising HepG2 Liver Carcinoma Inhibitors

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ABSTRACT: In one-pot strategy, diazotization of methyl anthranilate 5 followed by addition of amino acid ester hydrochloride, we have prepared methyl-2-(4-oxobenzotriazin-3(4H)-yl)-alkanoates 6a−c. Starting with hydrazides 7a,b, N-alkyl-2-(4-oxobenzotriazin-3(4H)-yl)-alkanamides 9−10(a−h) and methyl-2-(4-oxobenzotriazin-3(4H)-yl)alkanamido)alkanoates 11−12(a−e) were prepared via azide coupling. Hydrazones 13−15 were prepared via condensation of hydrazides 7a,b with 4-methoxybenzaldehyde, 4-dimethylaminobenzaldehyde, and/or arabinose. Molecular docking was done for synthesized compounds using MOE 2008-10 software. The compounds 9a, 12a, 12c, 13a, 13b, and 14b have the most pronounced strong binding affinities toward the target E. coli Fab-H receptor, whereas compounds 3, 11e, 12e, and 13a have the most pronounced strong binding affinities toward the target vitamin D receptor. The in vitro antibacterial activities of the highest binding affinity docked compounds were tested against E. coli, Staphylococcus aureus, and Salmonella spp. Majority of the tested compounds showed effective positive results against E. coli, while they were almost inactive against Staphylococcus aureus and Salmonella spp. The in vitro cytotoxic activities of the highest binding affinity-docked compounds were tested against the human liver carcinoma cell line (HepG2). Some compounds showed potent cytotoxic activity with low IC_{50} values, especially for 3 (6.525 μM) and 13a (10.97 μM) than that for standard drug doxorubicin (2.06 μM).

INTRODUCTION

Cancer is one of the major health challenges for all nations. Chemotherapy is one of the most effective targets used to heal malignancy. The main disadvantages of cancer chemotherapy are the dangerous side effects associated with it such as vomiting and spinal depression in addition to a lack of selectivity of drugs against the tumor cellular substance in the tumor compared to normal cellular substances. Hence, the search for anti-cancer drugs is the latest task that never ends. Benzotriazinones are key interactions at protein−protein interfaces that constitute important targets for small molecule inhibition because of their specific arrangements and biological importance. Therefore, one of the most corner stone principles in our research group is based on searching for new anticancer drugs. Benzotriazine and its derivatives possess a diverse range of biological activities of pharmacological activities including antimicrobial, anti-inflammaroty, anti-depressant, anti-ulcer, anti-diarrheal, anaesthesic, and anti-cancer. Some current commercial benzotriazinone anticancer drugs such as α-hydroxylated benzotriazinone (A), N-arylbenzotriazinones (B), tirapazamine (1,2,4-benzotriazin-3(4H)-one, which is one as a versatile reagent employed for peptide synthesis. Due to the importance of vitamin D, which has been produced by plants and animals, we used the vitamin D receptor in our docking investigation. VDR is found in almost all cells and tissues of higher-order animals, further emphasizing the importance of the receptor. Evidence for the existence of VDR was first provided in 1969 by Haussler and Norman, and since then a substantial amount of data on the structure and function of VDR has been accomplished.

In the present work, we aimed to synthesize a series of compounds containing benzotriazinone moieties on the basis of structure modification of methyl-2-(4-oxobenzotriazin-3(4H)-yl)alkanoates 6a−c for biological evaluation as antimicrobial and anticancer agents.

One of the most important benzotriazin-4(3H)-one derivatives is 3-hydroxy-1,2,3-benzotriazin-4(3H)-one, which is one as a versatile reagent employed for peptide synthesis (Figure 1). Due to the importance of vitamin D, which has been produced by plants and animals, we used the vitamin D receptor in our docking investigation. VDR is found in almost all cells and tissues of higher-order animals, further emphasizing the importance of the receptor. Evidence for the existence of VDR was first provided in 1969 by Haussler and Norman, and since then a substantial amount of data on the structure and function of VDR has been accomplished.
multistep reactions mentioned gave 6a in an overall high yield from available isatoic anhydride, but the alkyl halides needed to prepare other methyl-2-(4-oxobenzotriazin-3(4H)-yl)-alkanoates 6b–c were not available.

Diazotization of methyl antranilate 5 using sodium nitrite and HCl solution at 0 °C followed by addition of amino acid ester hydrochloride in the presence of triethylamine in a one-pot strategy afforded methyl-2-(4-oxobenzotriazin-3(4H)-yl)-alkanoates 6a–c in excellent yields, Scheme 2. This method has the advantage of simple work up, one pot, and the availability of reagents.

Scheme 2. Direct Preparation of Methyl 2-(4-Oxobenzotriazin-3(4H)-yl)alkanoates 6a–c and Corresponding Hydrazides 7a,b

The structure assignment of methyl-2-(4-oxobenzotriazin-3(4H)-yl)alkanoates 6a–c is based on full characterization, including 1H and 13C NMR spectroscopies. Thus, the 1H NMR spectrum of methyl-3-(4-oxobenzotriazin-3(4H)-yl)propanoate 6b revealed signals at δ 7.43–6.48, 3.65, and 2.95–2.89 ppm for OCH3, NCH3, and CH2CO groups, respectively. The 13C NMR spectrum of 6b showed signals at δ 171.0, 155.3, 51.9, 45.5, and 33.0 ppm for 2C=O groups, OCH3, NCH3, and CH2CO groups, respectively.

Methyl-2-(4-oxobenzotriazin-3(4H)-yl)alkanoates 6a–c are excellent precursors for structure modification of the benzotriazinone ring system via the azide coupling method by an attachment of either amines or amino acid through a peptide bond. Thus, the esters 6a,b were reacted with hydrazine hydrate in ethanol under reflux condition for 6 h afforded the corresponding hydrazides 7a,b, Scheme 2. Hydrazides 7a,b were reacted with NaNO2 and HCl in water at 0 °C for 1 h to afford the corresponding azides 8a,b and were extracted with ethyl acetate. The in situ generated azide 8a,b solution was successively added to primary amines iso-propyl, n-butyl, tert-butyl, n-decyl, cyclohexyl, and benzyl amines and secondary amines piperidine and morpholine to give N-alkyl-2-(4-oxobenzotriazin-3(4H)-yl)alkanamides 9–10(a–h) in good yields, Scheme 3.

Next, the in situ generated azide 8a and 8b solution was simultaneously added to amino acid methyl ester hydrochloride glycine, l-leucine γ-aminobutyric acid, β-alanine, and l-tryptophane to give methyl-2-(2-(4-oxobenzotriazin-3(4H)-yl)alkanamido)alkanoates (dipeptides) 11–12(a–e) in good yields via the azide coupling method, Scheme 4.

The structure assignments of N-alkyl-2-(4-oxobenzotriazin-3(4H)-yl)alkanamides 9–10(a–h) and methyl-2-(2-(4-oxobenzotriazin-3(4H)-yl)alkanamido)alkanoates (dipeptides) 11–12(a–e) are based on full characterization, including 1H and 13C NMR spectroscopies. Thus, the 1H NMR spectrum of methyl-2-(2-(4-oxobenzotriazin-3(4H)-yl)acetamido)acetate 11a showed signals at δ 5.22, 4.14, and 3.77 ppm corresponding to NCH3, NHCH2, and OCH3, respectively. The 13C NMR spectrum of 11a showed signals at 170.1, 155.3, 51.9, 45.5, and 33.0 ppm for 2C=O groups, OCH3, NCH3, and CH2CO groups, respectively.
52.5, and 41.4 ppm for 3C=O groups, OCH₃, NCH₂, and NHCH₂ groups, respectively.

Also, a number of hydrazones were prepared by condensation of hydrazides 7a,b with some aldehydes, namely, 4-methoxybenzaldehyde and 4-dimethylaminobenzaldehyde, in ethanol under reflux condition for 6 h and gave 13−14(a,b) in very good yields, Scheme 5.

Finally, the 3-(4-oxo-4H-benzo[d][1,2,3]triazin-3-yl)-propionic acid hydrazide 7b was condensed with arabinose in ethanol under reflux condition for 6 h and gave 15, Scheme 6.

Biological Activity. Molecular Docking Methodology. Bioinformatics including molecular modeling studies are very valuable at the present time in the field of drug discovery, saving money, and effort needed for the screening of new compounds by guiding and confining the investigation to possible target/ targets. The use of docking simulation studies in our work is quite important to help in predicting the possible mode of the action and structure activity relationship of the active derivatives and guiding the research future directions in the compound optimization and biochemical enzyme assay for the possible target enzymes. Key interactions at protein−protein interfaces constitute important targets for small molecule inhibition because of their specific arrangements and biological importance.

All the molecular modeling studies were carried out on Intel Core i3 CPU, 2.40 GHZ processor and 3 GB memory with a Windows 7 operating system using Molecular Operating Environment (MOE 2008-10 Chemical Computing Group, Canada) as the computational software. Anti-bacterial activities of the synthesized benzotriazinone derivatives were investigated through correlation with E. coli Fab-H inhibitory activities, and the anti-cancer activities of compounds were screened through detection of their abilities to act as the vitamin D receptor. The crystal structure of the E. coli FabH-CoA complex (PDB code: 1HNJ) and the crystal structure of the nuclear receptor for vitamin D bound to its natural ligand (PDB code: 1DB1) were obtained from the freely accessible protein data bank. The docking studies were performed after the verification process,
which was performed by redocking of the cocrystallized ligand into the active site using the default settings. The synthesized compounds were docked within the active sites of the crystallized structures using the MOE dock tool in MOE, performed with the default values. Different conformers for each compound are imported by systematic conformational of the MOE and saved in an mdb data base file to be docked into the active site of the receptor. Each complex was analyzed for the interaction, and 2D and 3D images were taken by using the MOE visualizing tool.

The results were evaluated based on binding affinity calculation together with cluster size determination and visually through the possible interaction with key residues at the active site.

**Anti-bacterial Activity. Molecular Modeling Study.** As shown in Figure 2, compound 12a formed two hydrogen bonds as the sidechain acceptor with amino acid residues Arg 36 and Arg 249. However, the amino acid residues Thr 28, Arg 36, and Arg 151 were not oriented directly to form complete hydrogen bonds with the ligand structure. Moreover, there are 16 ligand exposures, which indicate good binding affinity of the compound with the receptor under study.

As shown in Figure 3, compound 12c formed two hydrogen bonds as the side-chain acceptor with amino acid residues Asn 274 and Asn 247 in addition to one hydrogen bond as a backbone acceptor with the amino acid residue Gly 306. However, the amino acid residues Thr 28, Arg 36, and Arg 151 were not oriented directly to form complete hydrogen bonds with the ligand structure. Moreover, there are 10 ligand exposures, which indicate good binding affinity of the compound with the receptor under study.

As shown in Figure 4, compound 13b formed two hydrogen bonds as the sidechain acceptor with amino acid residues Asn 274. The amino acid residues Asn 247, Arg 36, and Thr 28 were not oriented directly to form complete hydrogen bonds with the ligand structure. Moreover, there are four ligand exposures, which indicate good binding affinity of the compound with the receptor under study.

**In Vitro Anti-bacterial Activity.** The in vitro antibacterial activities of the highest binding affinity-docked compounds 3,
7b, 9a, 10f, 11a, 12a, 12b, 12c, 13a, 13b, and 14b were tested against *E. coli*, *Staphylococcus aureus*, and *Salmonella* spp. Majority of tested compounds gave effective positive results against *E. coli* with an inhibitory zone of about 1.0 cm, while they were inactive against *Staphylococcus aureus* and *Salmonella* spp.

**Anti-cancer Activity. Molecular Modeling Study.**

As shown in Figure 5, compound 3 formed two hydrogen bonds as a side-chain acceptor with amino acid residues His 305 and His 379. However, the amino acid residues Tyr 143, Ser 237, Arg 274, and Ser 278 were not oriented directly to form complete hydrogen bonds with the ligand structure. Moreover, there are three ligand exposures, which indicate good binding affinity of the compound with the receptor under study.

As shown in Figure 6, compound 11e formed two hydrogen bonds as a side-chain acceptor with amino acid residues Ser 237 and Arg 274 in addition to one hydrogen bond as the arene–cation interaction with the amino acid residue Arg 274. Another hydrogen bond formed with the amino acid residue Tyr 143 as the arene–arene interaction. However, the amino acid residues Tyr 143, Ser 237, Ser 278, and His 305 were not oriented directly to form complete hydrogen bonds with the ligand structure. Moreover, there are four ligand exposures, which indicate good binding affinity of the compound with the receptor under study.

As shown in Figure 7, compound 13a formed two hydrogen bonds with the amino acid residue Tyr 143 one of them as a side-chain acceptor and the other one as the arene–arene interaction. In addition, two hydrogen bonds as a side-chain acceptor with the amino acid residue Ser 237. It also formed one hydrogen bond as the arene–arene interaction with the amino acid residue Trp 286. However, the amino acid residues Arg 274, Ser 278, His 305, and His 397 were not oriented directly to form complete hydrogen bonds with the ligand structure. Moreover, there are five ligand exposures, which indicate good binding affinity of the compound with the receptor under study.

**In Vitro Anti-cancer Activity.**

Potential cytotoxicity of the newly synthesized compounds was tested against the human liver carcinoma cell line (HepG2) using the method of Hansen et al. The in vitro anticancer screening was done by planting a tissue unit in Vacsera, Cairo A. R. Egypt. This work was performed by a modification (Hansen et al., 1989) of the tetrazolium salt (MTT) method, Mosmann, 1983. Preferably, cells should be plated in triplicate wells. Relative cell proliferation/viability was measured when treated cells are compared with untreated cells.
The in vitro cytotoxic activities of the highest binding affinity docked compounds were tested against human liver carcinoma (HepG2) cancer cell lines. Many compounds showed potent cytotoxic activities with low IC50 values, especially for 3 (6.525 μM) and 13a (10.97 μM), than that for standard drug doxorubicin (2.06 μM).

On regarding IC50 values, It is clear that the tryptophan amino acid fragments enhanced the benzotriazinone anticancer activities. Moreover, the hydrazone 13a derived from methoxy benzaldehyde in the case of glycine was more active than that of one derived from β-alanine.

**CONCLUSIONS**

A series of 36 compounds were synthesized based on structure modification of the model benzotriazinone 3 as a potent HepG2 liver carcinoma inhibitor. Different benzotriazinone derivatives were synthesized via different chemistry protocols to obtain 2-(4-oxobenzotriazin-3(4H)-yl)alkanoates methyl esters, 2-(4-oxobenzotriazin-3(4H)-yl)alkanamide derivatives, and 2-(2-(4-oxobenzotriazin-3(4H)-yl)alkanamido)alkanoates methyl esters. The hydrazones of some hydrazides were obtained by reactions with some aldehydes and/or arabinose. Applying of Molecular docking on synthesized compounds leads us to choose the most promising derivatives for further biological studies. Some compounds possess strong binding affinity toward the target E.coli Fab-H receptor. Moreover, some compounds had strong binding affinity toward the target vitamin D receptor. The in vitro antibacterial activities of the highest binding affinity docked compounds gave effective positive results against *E. coli*. On the other hand, the in vitro cytotoxic activities were tested against the human liver carcinoma cell line (HepG2), and IC50 values of compounds 3, 11e, 12e, and 13a were 6.525, 12.4019, 59.85, and 10.97μg/mL respectively.

**EXPERIMENTAL SECTION**

**General Procedure.** Thin-layer chromatography (TLC) was carried out on silica gel 60 F254 aluminum sheets (E. Merck, layer thickness 0.2 mm) in the following solvent systems, S1: petroleum ether/ethyl acetate (2:1) and S2: petroleum ether/ethyl acetate (1:1). The spots on thin layer plates were detected by the UV lamp. Melting points were determined on a Buchi 510 melting-point apparatus, and the values are uncorrected.

**Synthesis of Benzo[d][1,2,3]triazin-4(3H)-one (3).**

Method A: to an ice-cold solution of methyl anthranilate S (15.1 g, 0.1 mol) in diluted HCl contained in a 250 mL beaker, sodium nitrite (6.8 g, 0.116 mol) solution in water was added and the resulting diazonium solution was stirred at 0 °C for 5 min. Then, ammonium hydroxide was added dropwise until the solution becomes just alkaline. Amino acid ester hydrochlorides, glycine, β-alanine, and L-leucine (0.01 mol) were added dropwise, and the mixture was stirred for 5 min with subsequent neutralization by sodium carbonate. A precipitate was filtered off and washed with ice-cold ethanol. Product 6a–c was crystallized from ethanol.

Method B: a solution of benzo[d][1,2,3]triazin-4(3H)-one (3) (0.15 g, 1.0 mmol) in DMF (30 mL), K2CO3 (2.0 mmol), and methyl chloroacetate (0.12 mL, 1.0 mmol) was mixed and heated at 100 °C for 12 h while the reaction was monitored via TLC. The reaction mixture was then cooled, and an ice/water mixture was added. A precipitate out was filtered off, washed with ice-cold ethanol, and crystallized from ethanol.

**Methyl-2-(4-oxobenzotriazin-3(4H)-yl)alkanoates (6a–c).**

A yellow solid, (Method A, 69%), mp: 113–114 °C (Method B, 83%), (Lit. mp: 114–116 °C).23

**Methyl-3-(4-oxobenzotriazin-3(4H)-yl)-propanoate (6b).**

Oil (Method A, 87%), Rf = 0.75 (S2).1H NMR (300.0 MHz, CDCl3), δ ppm, (J, Hz): 8.28–8.23 (1H, m, Ar–H); 8.08–8.04 (1H, m, Ar–H); 7.91–7.86 (1H, m, Ar–H); 7.76–7.66 (1H, m, Ar–H); 4.73–4.68 (2H, m, NCH3); 3.65 (3H, s, OCH3); 2.95–2.89 (2H, m, CH2CO).13C-NMR (75 MHz, CDCl3), δ ppm, 171.0; 155.3(2CO); 144.1; 134.8; 132.4; 128.3; 125.0; 119.6 (Ar–C); 51.9 (OCH3); 45.5 (NCH3); 33.0 (CH2CO). MS (MALDI, positive mode, matrix DHB): m/z = 256 (M + Na)+. Anal. calc for C11H11N3O3 (233.2) C, 56.65; H, 5.61; N, 28.56. found C, 57.30; H, 3.37; N, 28.46.

**Methyl-4-(4-oxo-4H-benzo[d][1,2,3]triazin-3-yl)pentanoate (6c).**

White crystals (Method A, 84%), Rf = 0.32 (S2), mp: 197–200 °C.1H NMR (300.0 MHz, DMSO-d6), δ ppm, (J, Hz): 8.38–6.64 (4H, m, Ar–H); 5.82–5.74 (1H, m, NCHCO); 4.46–4.41 (2H, m, CH2); 3.97 (3H, s, OCH3); 2.53–1.65 (1H, m, CH); 0.97 (6H, d, J = 7.0 Hz, 2CH3). MS (MALDI, positive mode, matrix DHB): m/z = 298 (M + Na)+. Anal. calc for C15H21N3O7 (379.2) C, 56.65; H, 6.13; N, 15.21. Found C, 56.22; H, 6.13; N, 15.21.

**General Procedure for Syntheses of 2-(4-Oxo-3H)-1,2,3-benzotriazin-3-yl)alkanoic Acid Hydrazides 7a,b.**

To a solution of esters 6a,b (10.0 mmol) in ethanol (30 mL), hydrazine hydrate (3 mL, 48 mmol) was added. The reaction mixture was refluxed for 6 h; after cooling to room temperature, the precipitated hydrazide was filtered off, washed with water, and followed by recrystallization from aqueous ethanol.

2-(4-Oxo-3H)-1,2,3-benzotriazin-3-yl) Acetic Acid Hydrazide (7a). A white solid (85%), mp: 142–144 °C. Lit mp: 146–147 °C.
3-(4-Oxobenzo[d][1,2,3]triazin-3(4H)-yl)propanoic Acid Hydrazide (7b). Yellow crystals (80%), Rf = 0.38 (S2), mp: 165–168 °C. 1H NMR (300.0 MHz, CDCl3), δ ppm, (J, Hz): 9.11 (1H, s, NH); 8.27 (1H, d, J = 8.0 Hz, Ar−H); 8.21 (1H, d, J = 8.0 Hz, Ar−H); 8.12 (1H, t, J = 8.0 Hz, Ar−H); 7.96 (1H, t, J = 8.0 Hz, Ar−H); 4.61 (2H, t, J = 6.0 Hz, NCH3); 4.19 (2H, bs, NH2); 2.67 (2H, t, J = 6.0 Hz, CH2). 13C NMR (75 MHz, CDCl3), δ ppm, 169.2; 155.1 (2CO); 144.1; 135.8; 133.3; 128.4; 125.0; 119.7 (Ar−C); 46.5 (NCH3); 32.9 (CH2CO). MS (MALDI, positive mode, matrix DHB): m/z = 256 (M + Na)⁺. Anal. calc. for C13H13N3O2: C, 61.75; H, 5.92; N, 20.58. Found C, 61.71; H, 5.80; N, 20.68.

N-Isopropyl-2-(4-oxobenzo[d][1,2,3]triazin-3(4H)-yl)alkanamides (9a–9h). To a cold solution (−5 °C) of hydrazides 7a,b (8.0 ml) in acetic acid (60 ml), hydrochloric acid (5 N, 30 ml) was added portionwise under stirring a cold solution (0 °C) of sodium nitrite (0.7 g, 10.0 ml) in water (30 ml). After stirring at the same temperature for 30 min, the in situ generated azide 8a,b was extracted with cold ethyl acetate and washed successively with cold water, 5% NaHCO3 and water. After drying over anhydrous sodium sulfate, the azide 8a,b was used without further purification in the next step. Amines (9.0 ml) were added to the previously prepared cold dried solution of the azide 8a,b. Afterward, the mixture was kept for 12 h in the refrigerator and then at room temperature for another 12 h. The reaction mixture was washed with 0.1 N HCl, water, 5% NaHCO3 and water then dried over anhydrous sodium sulfate, the solvent was evaporated in vacuum and the residue was crystallized from ethyl acetate-petroleum ether to give products 9–10 (a–h).

N-Isopropyl-2-(4-oxobenzo[d][1,2,3]triazin-3(4H)-yl)acetamide (9a). White crystals (80%), Rf = 0.50 (S2), mp: 210–214 °C. 1H NMR (400.0 MHz, CDCl3), δ ppm, (J, Hz): 8.37 (1H, d, J = 8.0 Hz, Ar−H); 8.19 (1H, d, J = 8.0 Hz, Ar−H); 7.98 (1H, t, J = 7.6 Hz, Ar−H); 7.83 (1H, t, J = 7.6 Hz, Ar−H); 6.02 (1H, bs, NH); 5.07 (2H, s, NCH2CO); 4.15–4.10 (1H, m, CH); 1.60 (6H, d, J = 6.0 Hz, 2CH3). MS (MALDI, positive mode, matrix DHB): m/z = 269 (M + Na)⁺. Anal. calc. for C13H17N3O2: C, 65.30; H, 4.79; N, 19.04. Found C, 65.42; H, 4.65; N, 18.87.

N-Benzyl-2-(4-oxobenzo[d][1,2,3]triazin-3(4H)-yl)acetamide (9f). White crystals (77%), Rf = 0.37 (S2), mp: 187–190 °C. 1H NMR (300.0 MHz, CDCl3), δ ppm, (J, Hz): 8.36 (1H, d, J = 8.0 Hz, Ar−H); 8.20 (1H, d, J = 8.0 Hz, Ar−H); 8.00 (1H, t, J = 8.0 Hz, Ar−H); 7.85 (1H, t, J = 8.0 Hz, Ar−H); 7.37–7.30 (5H, m, Ar−H); 6.54 (1H, bs, NH); 5.14 (2H, s, NCH3); 4.51 (2H, d, J = 6.0 Hz, CHN2). 13C NMR (75 MHz, CDCl3), δ ppm, 166.0; 162.0 (COO); 144.3; 137.6; 135.2; 132.7; 130.8; 128.7 (Ar−C); 128.6; 128.3; 127.8; 127.0; 126.8 (Ar−C); 52.8 (NCH3); 43.9 (NCH3). (MALDI, positive mode, matrix DHB): m/z = 309 (M + Na)⁺. Anal. calc. for C19H17N3O2: C, 66.0; H, 6.25; N, 19.45.

N-Benzyl-2-(4-oxobenzo[d][1,2,3]triazin-3(4H)-yl)acetamide (9f). White crystals (85%), Rf = 0.36 (S2), mp: 240–242 °C. 1H NMR (400.0 MHz, CDCl3), δ ppm, (J, Hz): 8.30 (1H, d, J = 7.6 Hz, Ar−H); 8.13 (1H, d, J = 8.2 Hz, Ar−H); 7.92 (1H, t, J = 8.0 Hz, Ar−H); 7.77 (1H, t, J = 7.6 Hz, Ar−H); 5.81 (1H, bs, NH); 5.00 (2H, s, NCH2CO); 3.78–3.74 (1H, m, CH); 1.89–1.05 (10H, m, 5CH2). (MALDI, positive mode, matrix DHB): m/z = 309 (M + Na)⁺. Anal. calc. for C19H17N3O2: C, 66.0; H, 6.25; N, 19.45.
N-Butyl-3-(4-oxobenzo[d][1,2,3]triazin-3(4H)-yl)-propanamide (10b). White crystals (85%), Rf = 0.40 (S2), mp: 140–142 °C. 1H NMR (400.0 MHz, CDCl3), δ ppm, (J, Hz): 8.04 (1H, d, J = 7.6 Hz, Ar–H); 8.13 (1H, d, J = 8.0 Hz, Ar–H); 7.95 (1H, t, J = 7.6 Hz, Ar–H); 7.80 (1H, t, J = 7.6 Hz, Ar–H); 6.09 (1H, bs, NH); 4.79 (2H, t, J = 7.2 Hz, NCH3); 3.36–3.14 (2H, m, NHCH2); 2.87 (2H, t, J = 7.2 Hz, CH2CO); 1.47–1.40 (2H, m, CH2); 1.32–1.23 (2H, m, CH2); 0.88(3H, t, J = 7.2 Hz, CH3). (MALDI, positive mode, matrix DHB): m/z = 297 (M + Na+)Δ. Anal. calc'd for C16H20N4O2 (314.4) C, 66.02; H, 5.30; N, 19.34. Found C, 66.09; H, 5.24; N, 19.38.  

3-(3-Morpholino-3-oxopropyl)benzo[d][1,2,3]triazin-4(3H)-one (10h). White crystals (67%), Rf = 0.38 (S2), mp: 117–120 °C. 1H NMR (400.0 MHz, CDCl3), δ ppm, (J, Hz): 8.33–8.08 (1H, m, Ar–H); 8.14–8.11 (1H, m, Ar–H); 7.93–7.59 (1H, m, Ar–H); 7.53–7.48 (1H, m, Ar–H); 4.76–4.72 (2H, m, NCH3); 3.62–3.56 (4H, m, 2OCH2); 3.44–3.39 (4H, m, 2NCH2); 2.94–2.90 (2H, m, CH2CO). (MALDI, positive mode, matrix DHB): m/z = 311 (M + Na+)Δ. Anal. calc'd for C16H20N4O2 (288.3) C, 58.32; H, 5.59; N, 19.43. Found C, 58.12; H, 5.65; N, 19.31.

**General Procedure for Dipeptides**

Methyl 2-(2-(4-Oxobenzotriazin-3(4H)-yl)alkanamido)alkanoates (Dipeptides) (11–12(a–e)). To a cold solution (−5 °C) of hydrazides (7a,b) (1.6 mmol) in acetic acid (12 mL), hydrochloric acid (5 N, 6 mL) and water (50 mL) were added portionwise under stirring at a cold solution (0 °C) of sodium nitrite (0.14 g, 2 mmol) in water (6 mL). After stirring at the same temperature for 30 min, the azides 8a,b were extracted with cold ethyl acetate and washed successively with cold water, 5% NaHCO3, and water. After drying over anhydrous sodium sulfate, azides were used directly without further purification in the next step. Amino acid methyl ester hydrochlorides (1.8 mmol) were stirred in ethyl acetate (50 mL) with triethylamine (0.2 mL) at 0 °C for 20 min. The formed triethyl amine hydrochloride was filtered off, and the filtrate was added to the previously prepared cold-dried solution of the azide. Afterward, the mixture was kept for 12 h in the refrigerator and then at room temperature for another 12 h. The reaction mixture was washed with 0.1 N HCl water, 5% NaHCO3, and water then dried over anhydrous sodium sulfate. The solvent was evaporated in vacuum, and the residue was crystallized from ethyl acetate-petroleum ether to give the appropriate products 11–12(a–e).

Methyl-2-[(4-oxobenzo[d][1,2,3]triazin-3(4H)-yl)acetamidocaceta(n) (11a). White crystals (77%), Rf = 0.20 (S2), mp: 120–124 °C. 1H NMR (300.0 MHz, CDCl3), δ ppm, (J, Hz): 8.38 (1H, d, J = 8.0 Hz, Ar–H); 8.21 (1H, d, J = 8.0 Hz, Ar–H); 8.01 (1H, t, J = 8.2 Hz, NCH3); 7.69 (1H, t, J = 8.2 Hz, Ar–H); 7.79 (1H, t, J = 8.2 Hz, Ar–H); 6.79 (1H, bs, NH); 5.21 (2H, s, NCH2); 4.14 (2H, d, J = 6.0 Hz, CH2CO); 3.77 (3H, s, OCH3). 13C NMR (75 MHz, CDCl3), δ ppm, 170.1; 166.3; 155.9 (3CO); 144.3; 133.2; 135.7; 128.6; 125.1; 119.7 (Ar–C); 52.6 (NCH2); 52.5 (OCH3); 41.4 (NCH2CO) (MALDI, positive mode, matrix DHB): m/z = 296 (M + Na+)Δ. Anal. calc'd for C16H20N4O4 (368.5) C, 63.68; H, 8.87; N, 14.49. Found C, 63.80; H, 8.81; N, 14.52.

Methyl-2-[4-oxobenzo[d][1,2,3]triazin-3(4H)-yl]acetoaminocacetate (11b). White crystals (72%), Rf = 0.44 (S2), mp: 131–132 °C. 1H NMR (400.0 MHz, CDCl3), δ ppm, (J, Hz): 8.28 (1H, d, J = 8.0 Hz, Ar–H); 8.13 (1H, d, J = 8.0 Hz, Ar–H); 7.96–7.92 (1H, m, Ar–H); 7.80 (1H, t, J = 7.6 Hz, Ar–H); 7.34–7.22 (2H, m, Ar–H); 6.37 (1H, s, NH); 4.82 (2H, t, J = 7.2 Hz, NCH3); 4.44 (2H, d, J = 5.6 Hz, NHCH2); 2.92 (2H, t, J = 7.2 Hz, CH2CO). (MALDI, positive mode, matrix DHB): m/z = 331 (M + Na+)Δ. Anal. calc'd for C16H20N4O4 (304.4) C, 63.98; H, 6.71; N, 18.65. Found C, 64.09; H, 6.63; N, 18.59.

**Methyl-2-[4-oxo-4H-oxobenzo[d][1,2,3]triazin-3(4H)-yld]acetoaminobutanoate (11c).** White crystals (81%), Rf = 0.23 (S2), mp: 102–104 °C. 1H NMR (400.0 MHz, CDCl3), δ ppm, (J, Hz): 8.37 (1H, d, J = 7.8 Hz, Ar–H); 8.20 (1H, d, J = 7.8 Hz, Ar–H); 7.98 (1H, t, J = 7.8 Hz, Ar–H); 7.88 (1H, t, J = 7.8 Hz, Ar–H); 6.12 (1H, bs, NH); 5.07 (2H, s, NCH2); 3.70 (3H, s, OCH3); 3.33–3.31 (2H, m, NHCH2). 2.34 (2H, t, J = 7.2 Hz, CH2).
Methyl-3-[2-(4-oxobenzo[d]1,2,3)triazin-3(4H)-yl)-acetamidopropanooate (11d). White crystals (77%) , Rf = 0.35 (S), mp: 285–290 °C. 1H NMR (300.0 MHz, CDCl3), δ ppm, (J, Hz): 8.39 (1H, d, J = 8.0 Hz, Ar–H); 8.22 (1H, d, J = 8.0 Hz, Ar–H); 8.01 (1H, t, J = 7.6 Hz, Ar–H); 7.86 (1H, m, Ar–H); 5.87 (1H, bs, NH); 5.12 (2H, s, NCH2); 3.70 (3H, s, OCH3); 3.64–3.5 (2H, m, NCH2); 2.62 (2H, t, J = 6.8 Hz, CH2CO). (MALDI, positive mode, matrix DHB): m/z = 513 (M + Na)+.

Methyl-3-[2-(4-oxobenzo[d]1,2,3)triazin-3(4H)-yl)-propanamido)pentanoate (12b). White crystals (75%), Rf = 0.49 (S), mp: 138–140 °C. 1H NMR (400.0 MHz, CDCl3), δ ppm, (J, Hz): 8.32 (1H, d, J = 8.0 Hz, Ar–H); 8.15 (1H, d, J = 8.0 Hz, Ar–H); 7.97 (1H, t, J = 7.6 Hz, Ar–H); 7.82 (1H, t, J = 7.6 Hz, Ar–H); 6.35 (1H, d, J = 7.2 Hz, NH); 4.85–4.74 (2H, m, NCH2); 4.67–4.58 (1H, m, NCH2); 3.69 (3H, s, OCH3); 2.95 (2H, d, J = 6.8 Hz, CH2CO); 1.88 (1H, m, CH); 1.62–1.48 (2H, m, CH2); 0.89 (6H, t, J = 6.6 Hz, 2CH3). (MALDI, positive mode, matrix DHB): m/z = 369 (M + Na)+.

Methyl-3-[2-(4-oxobenzo[d]1,2,3)triazin-3(4H)-yl)propylenehydrazide (14a). White crystals (82%), Rf = 0.40 (S), mp: 226–229 °C. 1H NMR (400.0 MHz, CDCl3), δ ppm, (J, Hz): 8.99 (1H, s, CH); 8.71 (1H, d, J = 8 Hz, Ar); 8.33–6.62 (8H, m, NH & 2Ar–H); 5.63 (2H, s, NCH2CO); 2.97 (6H, s, 2NCH3). (MALDI, positive mode, matrix DHB): m/z = 373 (M + Na)+.

Methyl-3-[2-(4-oxobenzo[d]1,2,3)triazin-3(4H)-yl)propionylamino)propanoate (12d). White crystals (74%), Rf = 0.34 (S), mp: 285–290 °C. 1H NMR (300.0 MHz, CDCl3), δ ppm, (J, Hz): 8.37 (1H, d, J = 8.0 Hz, Ar–H); 8.26 (1H, d, J = 8.0 Hz, Ar–H); 7.98 (1H, m, Ar–H); 7.86 (1H, m, Ar–H); 6.05 (1H, bs, NH); 3.69 (3H, s, OCH3); 3.62–3.56 (2H, m, NCH2); 3.25–3.22 (2H, m, NCH2); 2.55 (2H, t, J = 6.0 Hz, CH2CO); 2.38 (2H, t, J = 6.6 Hz, CH2CO). (MALDI, positive mode, matrix DHB): m/z = 327 (M + Na)+.
Anal. calcd for C_{19}H_{20}N_{6}O_{2} (364.4) C, 62.62; H, 5.53; N, 19.89. Found C, 61.48; H, 4.97; N, 19.89.

N’-(4-(Dimethylamino)benzylidene)-3-(4-oxobenz[d]-1,2,3,4-triazin-3(4H)-yl)propanehydrazide (14b). White crystals (87.5%), cold ethanol, and recrystallized from aqueous ethanol. White mmol) was re.

Synthesis of 3-(4-Oxo-4H-benzo[d][1,2,3]triazin-3-yl)-proionic Acid (1,2,3,4,5-Pentahydroxy-pentylidene)-hydrazide (15). A mixture of hydrazide 7b (1.0 mmol) and arabinose (1.0 mmol) was refluxed in ethanol (20 mL) for 6 h. After cooling to room temperature, the resulting solid was filtered, washed with cold ethanol, and recrystallized from aqueous ethanol. White crystals (87.5%), Rf = 0.30 (S2), mp: 302 °C. 1H NMR (400.0 MHz, CDCl3), δ ppm, (J, Hz): 11.10 (1H, s, NH); 8.21–7.40 (4H, m, Ar−H); 5.43–4.24 (4H, m, 2CH2); 3.61–2.52 (10H, m, arabinose). (MALDI, positive mode, matrix DHB): m/z = 404 (M + Na)+. Anal. calcd for C_{15}H_{19}N_{5}O_{7} (381.3) C, 61.53; H, 4.88; N, 19.93.

EXPERIMENTAL PART OF BIOLOGY

Experimental Part for Antimicrobial Studies. The concentration was 200 ppm in DMF. Samples (20 μL each) were analyzed using “Disc diffusion method”1 against the following indicator strains:

a. E. coli: “Escherichia coli”

b. Staph: “Staphylococcus aureus (NCMB6571) (laboratory culture)”

c. Salmonella spp. typhimurium

Table 1. IC50 Values for Compounds 3, 11e, 12e, and 13a Tested on HEPG-2 Cell Line

| compound  | HEPG-2 IC50 (μg/mL) |
|-----------|---------------------|
| stand doxorubicin | 2.06 |
| 3          | 6.525               |
| 11e        | 12.4019             |
| 12e        | 59.85               |
| 13a        | 10.97               |

As shown in the Table 2, most of compounds more effective against E. coli in compared with staph and Salmonella spp. Most of compounds are in comparison with the starting material 3, which gave a negative result exposed a good activity against bacteria like; compounds 9a, 11a, 12a, 12b, 12c, 13a, 13b, and 14b. This data confirmed the identity between the experimental data and molecular docking data. Moreover, clear increasing for activity of the starting material benzotriazinone compound against bacteria gave a positive impression.

Experimental Part of Anti-cancer Activity (HEPG −2 Cell Line). In Vitro Anti-cancer Activity. Potential cytotoxicity of the newly synthesized compounds was tested against the human liver carcinoma cell line (HepG2) using the method of Hansen et al.19 The in vitro anticancer screening was done by planting a tissue unit in Vascera, Cairo A. R. Egypt.

| sample | E. coli | Staph | Salmonella spp. |
|--------|--------|-------|-----------------|
| control | –ve    | –ve   | –ve             |
| (3)     | –ve    | –ve   | –ve             |
| (7b)    | –ve    | –ve   | 1.0             |
| (9a)    | 0.9    | –ve   | –ve             |
| (10f)   | 0.7    | –ve   | –ve             |
| (11a)   | 1.0    | –ve   | 0.9             |
| (12a)   | 1.1    | –ve   | –ve             |
| (12b)   | 1.1    | –ve   | –ve             |
| (12c)   | 1.1    | –ve   | –ve             |
| (13a)   | 1.1    | –ve   | –ve             |
| (13b)   | 1.1    | –ve   | –ve             |
| (14b)   | 1.1    | –ve   | –ve             |

Table 2. Anti-bacterial Activity of the Compounds against E. coli, Staph, and Salmonella spp

This assay was performed by a modification (Hansen et al., 1989) of the tetrazolium salt (MTT) method (Mosmann, 1983).20 Preferably, cells should be planted in triplicate wells. Relative cell proliferation/viability was measured when treated cells are compared with untreated cells.

1. To each well of a 96-well flat-bottomed plate, add 25 μL of MTT (5 mg/mL stock solution).
2. Incubate 2 h at 37° C.
3. Add 100 μL of extraction buffer.
4. Incubate overnight at 37° C.
5. Read ODS570.
6. Include negative controls of no cells and extraction buffer as the blank.
7. Note: it is not necessary to remove medium prior to addition of reagents or to mix reagents in the well.

Reagent Preparation. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma cat. no. M2128) is dissolved at a concentration of 5 mg/mL in sterile PBS at room temperature. The filter sterilized and stored in a dark container at 4 °C. Prepare fresh each month, extraction buffer 20% (W/V) was dissolved at 37 °C in a solution of 50% DMF (N,N-dimethyl formamide; Fluka cat. no. 40250) and 50% SDW. Adjust pH to 4.7 by adding 2.5% of 80% acetic acid and 2.5% 1 N HCl.

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria. The MTT enters the cells and passes into the mitochondria

**Procedure.**

1. Plate cells (104−106 cells) in 200 mL of PBS in 96-well (flat bottom).
2. Add 20 mL of MTT solution, mix well.
3. Incubate for 4 h at 37 °C in dark.
4. Remove the aliquot for analysis, add 200 mL acidic isopropanol, and mix well.
5. Incubate additional 1 h in dark at 37 °C.
6. Read plate in the ELISA reader, measure OD in 570 nm (the background wavelength is 630 nm).

As shown in the Table 1, the compound 3 shown an IC_{50} of 6.525 μg/mL whereas the derivatives 11e shown an IC_{50} of 12.40 μg/mL, 13a shown an IC_{50} of 10.97 μg/mL, and compound 12e shown an IC_{50} of 59.85 μg/mL. In comparing with the Doxorubicin standard IC_{50} of 2.06 μg/mL. We found that the starting material benzo[1,4]triazinone 3 was the highest active compound having an IC_{50} of 6.525 μg/mL; this result showed that the starting material compound 3 need more potent that is less likely to cause resistance and recurrence of cancer and less toxic to normal tissues than available chemotherapeutic agents.

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**Notes**

The authors declare no competing financial interest.

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