Synthesis, Antitumor and Antiviral In Vitro Activities of New Benzotriazole-Dicarboxamide Derivatives

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Cancer and viral infections continue to threaten humankind causing death worldwide. Hence, the discovery of new anticancer and antiviral agents still represents a major scientific goal. Heterocycles designed to mimic the chemical structure of natural pyrimidines and purines have been designed over the years, exerting their activity acting as false substrates on several different targets. We reported a series of bis-benzotriazole-dicarboxamide derivatives which inhibit viral helicase of poliovirus, and hence we planned structure modifications to obtain different series of new dicarboxamides. Here, the synthesis and characterization of 56 new compounds: 31 bis-benzotriazole dicarboxamides and 25 mono-substituted acidic derivatives are reported. The synthesized compounds were tested for their antiviral and antitumor activity. Mostly, compounds 4a, 4c and 4d showed antiviral activity against tested Picornaviruses, Coxsackievirus B5 and Poliovirus-1. Likewise, four derivatives (3b, 3d, 4d, 9b) showed notable antiproliferative activity inhibiting cell growth in two distinct antitumor screenings. Compound 3b was selected as the antitumor lead compound for the wide range of activity and the potency proved. The lead compound was proved to induce apoptosis in SK-MES1 tumor cells, in a dose-dependent manner.

Keywords: antimetabolites, antiproliferative activity, antivirals, benzotriazole derivatives, dicarboxamides, coxsackievirus

INTRODUCTION

Among the various diseases that affect humankind, cancer and viral infections remain great threats to human health worldwide. Therefore, the identification of novel anticancer and antiviral agents remains one of the most pressing health problems and the most stimulating cue for research. Polycyclic benzo-fused azoles can mimic the chemical structure of natural pyrimidines and purines, exerting their biological activity acting as false substrates, resulting in antitumor, antibacterial or antiviral agents. Considering the similar mechanism of action of the latter, cross-activity has been highlighted for several molecules (Kaufman, 1980; Matsuda et al., 1999; Ramachandran et al., 2011; Brüning et al., 2012). Therefore, anticancer drugs have been proved active against viruses (Kaufman, 1980) and antiviral agents have shown antimetabolite activity (Bergman et al., 2002; De Clercq, 2004;
Brüning et al., 2012). 5-fluorouracil is a well-known example; it is a widely used anticancer agent which acts as antimetabolite. It was proved active against several viruses such as foot and mouth disease virus (Pariente et al., 2003; Agudo et al., 2009), herpes simplex virus (Dragún et al., 1990), poliovirus (Cooper, 1964). Purine-like antimetabolites enter the metabolic pathway acting as false substrates or mimicking the natural building blocks of RNA and DNA (De Clercq, 2013). For these features, besides human-cell targets, viral enzymes involved in RNA and DNA synthesis, such as RNA-dependent-RNA-polymerases (RdRp) (Furuta et al., 2017), helicases (Carta et al., 2007), proteases (Kneller et al., 2019), may be targeted by this class of derivatives. Benzotriazole (BT) derivatives have been widely studied for their broad biological activity (Briguglio et al., 2015). BT derivatives may mimic purine ring acting as antimetabolites interfering with purine metabolism but may also bind the enzymes that naturally interact with purines, mimicking the natural substrate, resulting in potential antitumor (Al-Soud et al., 2003), antibacterial (Sanna et al., 1992; Carta et al., 2011; Ochal et al., 2013), antifungal (Patel et al., 2010) or antiviral (Borowski et al., 2003) agents. In the process of new drug discovery, we designed several promising antiviral active derivatives bearing the benzotriazole scaffold (Ibba et al., 2018; Piras et al., 2019a; Piras et al., 2019b; Sanna et al., 2020). Amongst them, benzotriazole derivatives depicted in Figure 1 have been designed, synthesized and proved as antiviral agents against two tested Picornaviruses (Coxsackievirus B2 and Poliovirus Sb-1) (Carta et al., 2007). Bis-benzotriazole-dicarboxamide derivatives (Figure 1) were proved to exert their antiviral activity as false substrates binding the Poliovirus helicase in in silico assays, performed on the 3D model of the target protein (Carta et al., 2007). Bis-benzotriazole-dicarboxamide derivatives from series 2 turned out as the most active with EC50 values ranging from 4 to 33 μM against Coxsackievirus B2.

Based on this, we designed and synthesized a new series of bis-benzotriazole-dicarboxamide derivatives as potential anti-Picornavirus agents, acting as false substrates. The structural modifications applied to the main scaffold are shown in red in Figure 2. We evaluated the substitution on the benzotriazole scaffold in positions 5 and 6 (R). We also considered the length and the role of the linker amidst the two amidic groups (X), including saturated or unsaturated aliphatic or small aromatic linkers. The insertion of a CH2 bridge between the BT scaffold and the aromatic moiety directly connected to the BT nitrogen was also attempted.

All the synthesized compounds were tested against a panel of Picornaviruses and several cell lines to assess the cytotoxicity. Only a few of the designed molecules turned out active against tested viruses, while some were selected for antiproliferative assays, based on the known antiviral-anticancer correlations. Notably, four derivatives (3b, 3d, 4d, 9b) showed interesting antiproliferative activity inhibiting cell growth and inducing apoptosis. Compound 3b was selected as the lead compound to be subjected to further structure-modifications in order to improve the antiproliferative activity.

**MATERIALS AND METHODS**

**Chemistry**

Reaction progression, retention factors (Rf) and purity of compounds were monitored by TLC using Merck F-254 commercial plates and a proper mixture of solvents as eluent. Melting points were taken in open capillaries in a Köfler hot stage or Digital Electrothermal melting point apparatus. 1H-NMR spectra were determined in DMSO-d6 and were recorded with a Bruker Avance III 400 NanoBay (400 MHz) and a Varian XL-200 (200 MHz) instruments. Chemical shifts (δ scale) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) used as internal standard. The chemical shift values are reported in ppm (δ) and coupling constants (J) in
fraction with b.p. 40 mesh (Merck silica gel 60). Petroleum ether (PE) refers to the fraction with b.p. 40–60°C. Elemental analysis was performed on a Perkin-Elmer 2400 instrument at "Laboratorio di Microanalisi", Department of Chemistry and Pharmacy, University of Sassari, Italy, and the results were within ±0.4% of theoretical values.

Biology

Cells and Viruses

Cell lines were purchased from American Type Culture Collection (ATCC). Cell lines supporting the multiplication of CV-B5 and Sb-1 are Monkey kidney (Vero-76) [ATCC CRL 1587 Cercocephemus Aethiops]. Viruses were purchased from the American Type Culture Collection (ATCC). Picornaviridae: Coxackievirus [coxsackie type B5 (CV-B5), strain Faulkner (ATCC VR-185)], and human enterovirus C [poliovirus type-1 (Sb-1), Sabin strain Chat (ATCC VR-1562)]. Cell cultures were checked periodically for the absence of mycoplasma contamination with MycoTect Kit (Gibco). Viruses were maintained in our laboratory and propagated in appropriate cell lines. The viruses were stored in small aliquots at −80°C until use.

Cytotoxicity Assays

Vero-76 cells were seeded in 96-well plates at an initial density of 5 × 10³ cells/ml, in Dulbecco’s Modified Eagle Medium (D-MEM) with L-glutamine and 25 mg/L kanamycin, supplemented with 10% FBS. Cell cultures were then incubated at 37°C in a humidified and 5% CO₂ atmosphere, in the absence or presence of serial dilutions of test compounds. The test medium used for the cytotoxic assay as well as for antiviral assay contained 1% of the appropriate serum. Cell viability was determined after 48–96 h at 37°C by MTT method for Vero-76 (Pauwels et al., 1988).

Antiviral Assays

Compounds activity against CV-B5 and Sb-1 was determined by plaque reduction assays in infected cell monolayers. Briefly, monolayer of Vero-76 cells was grown overnight on 24-well plate. The cells were then infected for 2 h with 250 μl of proper virus dilutions to give 50–100 PFU/well. Following removal of unadsorbed virus, 500 μl of medium (D-MEM with L-glutamine and 4,500 mg/L D-glucose, supplemented with 1% inactivated FBS) containing 0.75% methyl-cellulose, with serial dilutions of test compounds, were added. The overlayed medium was also added to not treated wells as non-infection controls. Cultures were incubated at 37°C for 2 days for Sb-1 and 3 days for CV-B5 and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed and air-dried. Plaques in the control (no inhibitor) and experimental wells were then counted (Sanna et al., 2015).

Antiproliferative Assays

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. Exponentially growing leukemia and lymphoma cells were seeded at 1 × 10⁵ cells/ml in 96 well plates in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin, and incubated at 37°C in a humidified, 5% CO₂ atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 96 h at 37°C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. Activity against solid-tumor derived cells was evaluated in exponentially growing cultures seeded at 5 × 10³ cells/ml and allowed to adhere for 16 h to culture plates before the addition of the drugs. Cell viability was determined by the MTT method 4 days later. Vincristine and doxorubicin were used as reference drugs.

Linear Regression Analysis

The extent of cell growth/viability and viral multiplication, at each drug concentration tested, were expressed as percentage of untreated controls. Concentrations resulting in 50% inhibition (CC₅₀ or EC₅₀) were determined by linear regression analysis.

NCI60 Human Tumor Cell Lines Screen

The in vitro anti-cancer screening was performed through the NCI60 Human Tumor Cell Lines Screen, provided by the Developmental Therapeutics Program of the National Cancer Institute (NCI, Bethesda, United States). A single dose of 10 μM of each compound is tested in the whole NCI60 cell panel. More details about the NCI60 screen methods are reported on the NCI website (https://dtp.cancer.gov).

Apoptosis Assay

To assess which death mechanisms our compounds induced, cell apoptosis kit Annexin V/Propidium iodide (PI) double staining uptake (Invitrogen, Life Technologies, Italy) was used. 3b compound was selected as lead and employed in our assay. Human lung cancer cells (SK-MES 1) were seeded at the density of 8 × 10⁵ cells/ml in 6-well plates (Corning, United States) with a complete medium (described in cell culture section). After overnight incubations, the cells were treated with or without different concentrations of 3b for 96 h. Cells were then labeled with Annexin V and PI as previously described (Ibba et al., 2021). Stained cells were then analyzed by flow cytometry, measuring the fluorescence emission at 530 and 620 nm using 488 nm excitation laser (MoFloAstrios EQ, Beckman Coulter). Cell apoptosis was analyzed using Software Summit Version 6.3.1.1, Beckman Coulter.

Experimental Intermediates

5,6-Dichloro-1H-benzo[d][1,2,3]triazole (21) was synthesized as previously described (Carta et al., 2007), while 1H-Benzо[d][1,2,3]triazole 19, 5,6-Dimethyl-1H-benzo[d][1,2,3]triazole 20,
1- (Chloromethyl)-4-nitrobenzene and the diacyl dichlorides (14a-g) were commercially available. The intermediate 1 (2)- (4-Aminophenyl)-5,6-dichlorobenzonitrile [d][1,2,3]triazoles (13 and 15) were prepared according to the procedures previously described (Carta et al., 2007).

**General Procedure to Obtain (5,6-R)-1-(4-Nitrobenzyl)-1H-benzo[d][1,2,3]triazole (22-24)**

To a mixture of proper benzotriazole (19) or 5,6-Dimethylbenzotriazole (20) or 5,6-Dichlorobenzotriazole (21) (2.7 mmol) and Cs2CO3 (2.7 mmol) in 30 ml of N,N-Dimethylformamide (DMF) a solution of 4-Nitrobenzylchloride (5.4 mmol) in 10 ml of DMF was added. The mixture was heated to 60°C for 48 h. After cooling down, the solution was filtered off when the solution was still hot, and the mothers were diluted with water until complete precipitation of R two isomers: 5,6-(400 MHz, CDCl3) δ 7.51 (2H, d, J = 8.6, H-2′,6′), 6.03 (2H, s, CH2). 13C-NMR (DMSO-d6) δ: 145.9 (C), 145.3 (C), 142.5 (C), 133.8 (2C), 132.6 (C), 128.6 (2CH), 126.5 (2CH), 124.3 (2CH), 52.0 (CH2).

**Compounds**

- 4-((5,6-Dimethyl-1H-Benzo[d][1,2,3]triazol-1-yl)methyl)aniline (16)
- 4-((5,6-Dichloro-1H-Benzo[d][1,2,3]triazol-1-yl)methyl)aniline (18)

4-((1H-Benzo[d][1,2,3]Triazol-1-yl)methyl)aniline (16)

To a mixture of proper benzotriazole 22 or 23 (1.77 mmol) in Ethanol (20 ml), and Hydrated hydrazine in ratio 1:20 (35.4 mmol), Pd/C in ratio 1:0.1 w/w was added. The mixture was stirred and heated at 80°C for 1 h. Palladium on carbon was filtered off when the solution was still hot, and the mothers obtained were concentrated into half volume. By cooling down, the resulting solid was filtered out in vacuo. The solid compound was washed twice with diethyl ether (20 ml) and subsequently crystallized from Ethanol. Derivative 24 (1.77 mmol) was solved in Ethanol (100 ml) and reduced with Methylhydrazine in 1/10 molar ratio, at 100°C in autoclave for 48 h.

4-((5,6-Dimethyl-1H-Benzo[d][1,2,3]triazol-1-yl)methyl)aniline (17)

Compounds 16 was obtained in 78% total yield; m.p. 151–153°C; TLC (petroleum ether/ethyl acetate 4/6); Rf = 0.47; 1H-NMR (200 MHz, DMSO-d6) δ: 8.03 (1H, d, J = 8.4, H-4), 7.81 (1H, d, J = 8.4, H-7), 7.50 (1H, t, H-5), 7.41 (1H, t, H-6) 7.07 (2H, d, J = 8.2, H-3′,5′), 6.49 (2H, d, J = 8.2, H-2′,6′), 5.74 (2H, s, CH2), 5.15 (2H, s, NH2). 13C-NMR (DMSO-d6) δ: 149.2 (C), 145.5 (C), 132.4 (C), 132.8 (C), 128.7 (2CH), 126.6 (2CH), 119.7 (CH), 114.6 (2CH), 110.3 (CH), 52.3 (CH2).

4-((5,6-Dichloro-1H-Benzo[d][1,2,3]triazol-1-yl)methyl)aniline (18)

Compounds 18 was obtained in 30% total yield; m.p. 203–206°C; TLC (petroleum ether/ethyl acetate 7/3); Rf = 0.29; 1H-NMR (400 MHz, CDCl3) δ: 8.45 (1H, s, H-4), 8.32 (1H, s, H-7), 7.11 (2H, d, J = 8.4, H-2′,6′), 6.51 (2H, d, J = 8.4, H-3′,5′), 5.75 (2H, s, CH2), 5.13 (2H, s, NH2). 13C-NMR (DMSO-d6) δ: 145.7 (C), 144.9 (C), 133.8 (2C), 133.0 (C), 129.5 (2CH), 126.6 (2CH), 125.9 (C), 115.4 (2CH), 52.3 (CH2).

**General Procedure to Obtain Final Derivatives 3a-g and 4a-d,f; 5e-g and 6f; 7a-g and 10a-g; 8a-g and 11a-g; 9a-g and 12a-d,f**

To a solution of 0.3 g (1.19 mmol) of intermediates 13, 15, or 16-18 and Triethylamine 0.17 ml (1.19 mmol) dissolved in 9 ml...
of DMF, the appropriate acyl dichloride 14a-g (1.44 mmol, ratio 1:1.2) was added. The mixture was left to react at rt. under stirring for a varying time from 3 to 96 h (3 h for compounds 3g, 5e, 7a-g, 9a-e) 9 h for compounds 3b, 9b,c 24 h for compounds 3c,e, 5g, 8a-e, 9f,g 48 h for compounds 5f, 9d; 72 h for compounds 3a,d,f; 96 h for compounds 8f,g. Then, Triethylamine hydrochloride was removed by filtration with vacuum and the mother liquid was diluted with water to obtain a precipitate, which consisted of a mixture of the two products: the bis-benzotriazole dicarboxamides (series 3, 5, 7, 8, 9) and the corresponding mono-substituted acids (series 4, 6, 10, 11, 12). The separation of the two compounds (dimer and corresponding acid) was obtained by flash chromatography using a mixture of chloroform and methanol (ratio 9:8:0.2) as eluent for the pairs 7/10 and 8/11 in which the dimer derivatives is the first to be eluted and represents only 1/3 of the mixture, while derivatives bearing two chlorine atoms in the benzotriazole moiety (pairs 3/4, 5/6, and 9/12) were separated by repeated washing of the solid on a porous silica gel (60°C) mixture of chloroform, acetone and methanol in a 6:3:1 ratio. The mono-substituted acidic derivatives (4, 6, 12) were solved by the mixture and thereafter recovered by evaporation in vacuo of the solvent and further purified by fractional crystallization using ethyl acetate and petroleum ether. The dicarboxamide derivative, instead, remained unsolved on the filter was further purified by repeated washings with acetone.

$N^4,N^6$-Bis(4-(5,6-Dichloro-1H-Benzo[d][1,2,3]Triazol-1-yl)phenyl)succinamide (3a)

Compound 3a was obtained in 20% total yield; m.p. 264–265°C; TLC (chloroform/methanol 9.5:0.5); Rf = 0.84; $^1$H-NMR (400 MHz, DMSO-d$_6$) δ: 10.43 (2H, s, NH), 8.58 (2H, s, H-4), 8.44 (2H, s, H-7), 7.91 (4H, d, $J$ = 8.0, H-3′,5′), 7.86 (4H, d, $J$ = 8.0, H-2′,6′), 2.78 (4H, s, CH$_2$-CH$_3$). $^{13}$C-NMR (DMSO-d$_6$) δ: 170.8 (2CO), 144.6 (C) 143.2 (C), 140.9 (C), 140.1 (2C), 133.9 (C), 131.8 (C), 131.1 (C), 130.3 (2C), 127.6 (2C), 126.3 (4CH), 121.1 (CH), 120.8 (CH), 119.8 (2CH), 119.5 (CH), 119.4 (CH), 112.8 (2CH), 31.2 (CH$_2$), 28.7 (CH$_2$). C$_{20}$H$_{16}$Cl$_2$N$_2$O$_2$; MW 460.31; Elem. Anal.: Calcd C 53.91, H 3.96, N 19.49, Br 10.31, Cl 11.92. LC/MS: m/z 661 [M + Na$^+$], 641 [M + H]$^+$, 639 [M + H]$^+$.

$N^4,N^6$-Bis(4-(5,6-Dichloro-1H-Benzo[d][1,2,3]Triazol-1-yl)phenyl)adipamide (3c)

Compound 3c was obtained in 15% total yield; m.p. 293–294°C; TLC (chloroform/methanol 9.5:0.5); Rf = 0.62; $^1$H-NMR (400 MHz, DMSO-d$_6$) δ: 10.28 (2H, s, NH), 8.60 (2H, s, H-4), 8.22 (2H, s, H-7), 7.90 (4H, d, $J$ = 8.0, H-3′,5′), 7.88 (4H, d, $J$ = 8.0, H-2′,6′), 2.44 (4H, s, CH$_2$COOH), 1.23 (4H, s, CH$_2$-CH$_2$). $^{13}$C-NMR (DMSO-d$_6$) δ: 171.5 (2CO), 144.6 (C), 143.2 (C), 140.9 (C), 140.1 (2C), 134.0 (C), 131.8 (C), 131.1 (C), 130.3 (2C), 127.6 (2C), 126.3 (4CH), 121.1 (CH), 120.8 (CH), 119.8 (2CH), 119.5 (CH), 119.4 (CH), 112.8 (2CH), 36.3 (2CH$_2$), 24.8 (2CH$_2$). C$_{30}$H$_{22}$Cl$_4$N$_8$O$_2$; MW 668.36; Elem. Anal.: Calcd C 53.91, H 3.96, N 19.49, Br 10.31, Cl 11.92. LC/MS: m/z 671 [M + Na$^+$], 669 [M + H]$^+$, 667 [M + H]$^+$.

$N^4,N^6$-Bis(4-(5,6-Dichloro-1H-Benzo[d][1,2,3]Triazol-1-yl)phenyl)glutaramide (3b)

Compound 3b was obtained in 22% total yield; m.p. 266–267°C; TLC (chloroform/methanol 9.5:0.5); Rf = 0.62; $^1$H-NMR (400 MHz, DMSO-d$_6$) δ: 10.30 (2H, s, NH), 8.61 (2H, s, H-4), 8.25 (2H, s, H-7), 7.92 (4H, d, $J$ = 8.0, H-3′,5′), 7.82 (4H, d, $J$ = 8.0, H-2′,6′), 2.51 (4H, s, CH$_2$CO), 2.01 (2H, t, -CH$_2$-). $^{13}$C-NMR (DMSO-d$_6$) δ: 171.2 (2CO), 144.6 (2C), 140.1 (2C), 131.8 (2CH), 131.1 (2C), 130.3 (2CH), 127.6 (4CH), 120.8 (2CH), 119.9 (4CH), 112.8 (2CH), 35.6 (2CH$_2$), 20.7 (CH$_2$). C$_{26}$H$_{22}$Cl$_4$N$_8$O$_2$; MW 654.33; Elem. Anal.: Calcd C 53.23, H 3.08, N 17.12 Found C 52.93, H 3.35, N 17.28. LC/MS: m/z 677 [M + Na]$^+$, 657 [M + H]$^+$, 653 [M + H]$^+$.
Ibba et al.\footnote{\textbf{Synthesis Antitumor Antiviral Activities Benzotriazoledicarboxamide}}

\[ \text{N}^1,\text{N}^2,\text{N}^4,\text{N}^6\text{-Bis}(\text{4-}(5,6\text{-Dichloro}-1\text{-H}-\text{Benzo}[d][1,2,3]\text{Triazol-1-yl})\text{phenyl})\text{furamamide (3d)} \]

Compound 3d was obtained in 18% total yield; m.p. >300°C; TLC (chloroform/methanol 9.5:0.5); Rf = 0.90; 1H-NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 10.92 (1H, s, NH), 8.64 (2H, s, H-4), 8.32 (2H, s, H-7), 8.02 (4H, \(d\), \(J = 8.4, H-3′,5′\)), 7.91 (4H, \(d\), \(f = 8.4, H-2′,6′\)), 7.31 (2H, s, CH=CH). 13C-NMR (DMSO-\(d_6\)) \(\delta\) 162.3 (2CO), 144.7 (2C), 141.5 (2C), 139.5 (2C), 134.8 (2C), 134.2 (CH=CH), 131.1 (2C), 127.7 (2C), 123.8 (4CH), 120.9 (2CH), 120.5 (4CH), 112.9 (2CH). C\(_{19}\)H\(_{11}\)Cl\(_2\)N\(_5\)O\(_3\); MW 689.33; Elem. Anal.: Calcld C 53.54, H 2.49, N 16.28 Found C 53.54, H 2.50, N 16.45. LC/MS: m/z 690 [M + H]\(^+\), 692 [M + H]\(^+\). \(6-((4-\text{((5,6\text{-Dichloro}-1\text{-H}-\text{Benzo}[d][1,2,3]\text{Triazol-1-yl})\text{phenyl})\text{carbamoyl})\text{picolinic Acid (4f)}}\)

Compound 4f was obtained in 30% total yield; m.p. 240°C; TLC (chloroform/methanol 9/1); Rf = 0.1; 1H-NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 11.39 (1H, s, OH), 11.16 (1H, s, NH), 8.80 (1H, s, H-4), 8.58 (1H, \(t\), \(H-4′\)), 8.31 (5H, s, H-7, H-2′,3′,5′,6′), 8.16 (1H, \(d\), \(J = 8.0, H-3′\)), 7.98 (1H, \(d\), \(J = 8.0, H-5′\)). 13C-NMR (DMSO-\(d_6\)) \(\delta\) 164.9 (CO), 161.9 (CO), 148.9 (C), 146.7 (C), 144.7 (C), 140.1 (CH), 138.8 (C), 131.9 (C), 131.5 (C), 131.0 (2C), 127.7 (2C), 125.8 (CH), 123.5 (CH), 123.4 (CH), 121.8 (CH), 121.6 (CH), 120.9 (CH), 113.0 (CH). C\(_{19}\)H\(_{11}\)Cl\(_2\)N\(_5\)O\(_3\); MW 428.22; Elem. Anal.: Calcld C 53.53, H 2.59, N 16.56 Found C 53.39, H 2.79, N 16.25. LC/MS: m/z 429 [M + H]\(^+\), 427 [M + H]\(^+\). \(6\text{-}((4-\text{((5,6\text{-Dichloro}-1\text{-H}-\text{Benzo}[d][1,2,3]\text{Triazol-1-yl})\text{phenyl})\text{carbamoyl})\text{picolinic Acid (4f)}}\)

\(N^2,N^5\text{-Bis}(\text{4-}5\text{6-Dichloro}-1\text{-H-Benzo}[d][1,2,3]\text{Triazol-1-yl})\text{phenyl})\text{thiophene-2,5-dicarboxamide (3g)}\)

Compound 3g was obtained in 15% total yield; m.p. 208–209°C; TLC (chloroform/methanol 9/1); Rf = 0.87; 1H-NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 10.75 (2H, s, NH), 8.64 (2H, s, 2H-4), 8.34 (2H, s, 2H-7), 8.12 (2H, \(d\), \(J = 8.0, H-3′,4′\)), 8.08 (4H, \(s\), 2H-2′,6′), 7.93 (4H, \(s\), 2H-3′,5′). 13C-NMR (DMSO-\(d_6\)) \(\delta\) 159.6 (CO), 159.5 (CO), 144.7 (2C), 143.8 (2C), 139.3 (2C), 131.9 (2C), 131.3 (2C), 129.8 (2CH), 127.7 (2C), 126.3 (4CH), 121.4 (4CH), 120.9 (2CH), 112.9 (2CH). C\(_{19}\)H\(_{11}\)Cl\(_2\)N\(_5\)O\(_3\); MW 694.37; Elem. Anal.: Calcld C 51.90, H 2.32, N 16.14 Found C 51.90, H 2.32, N 16.14. LC/MS: m/z 495 [M + H]\(^+\), 493 [M + H]\(^+\). \(6\text{-}((4-\text{((5,6\text{-Dichloro}-1\text{-H}-\text{Benz}[d][1,2,3]\text{Triazol-2-yl})\text{phenyl})\text{carbamoyl})\text{picolinic Acid (4g)}}\)

\(N^2,N^6\text{-Bis}(\text{4-}5\text{6-Dichloro}-2\text{-H-Benzo}[d][1,2,3]\text{Triazol-2-yl})\text{phenyl})\text{thiophene-2,5-dicarboxamide (3g)}\)

Compound 3g was obtained in 15% total yield; m.p. 208–209°C; TLC (chloroform/methanol 9/1); Rf = 0.87; 1H-NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 10.75 (2H, s, NH), 8.64 (2H, s, 2H-4), 8.34 (2H, s, 2H-7), 8.12 (2H, \(d\), \(J = 8.0, H-3′,4′\)), 8.08 (4H, \(s\), 2H-2′,6′), 7.93 (4H, \(s\), 2H-3′,5′). 13C-NMR (DMSO-\(d_6\)) \(\delta\) 159.6 (CO), 159.5 (CO), 144.7 (2C), 143.8 (2C), 139.3 (2C), 131.9 (2C), 131.3 (2C), 129.8 (2CH), 127.7 (2C), 126.3 (4CH), 121.4 (4CH), 120.9 (2CH), 112.9 (2CH). C\(_{19}\)H\(_{11}\)Cl\(_2\)N\(_5\)O\(_3\); MW 694.37; Elem. Anal.: Calcld C 51.90, H 2.32, N 16.14 Found C 51.90, H 2.32, N 16.14. LC/MS: m/z 495 [M + H]\(^+\), 493 [M + H]\(^+\).
**N²,N⁶-Bis-(4-(5,6-Dichloro-2H-Benz[d][1,2,3]Triazol-2-yl)phenyl)pyridine-2,6-dicarboxamide (5f)**

Compound 5f was obtained in 15% total yield; m.p. >300°C; TLC (chloroform/methanol 9/1); RF = 0.1; ¹H-NMR (400 MHz, DMSO-d₆): δ: 8.16 (4H, d, 2H-5). 8.32 (4H, d, 2H-4), 7.76 (4H, d, J = 8.8, 2H-3'), 7.67 (4H, d, J = 8.8, 2H-2'), 7.51-7.48 (2H, m, 2H-5'), 7.39 (2H, t, H-6). 7.27 (4H, d, J = 8.4, 2H-3', 2H-5'). 5.90 (4H, s, 2CH₂). 2.59 (4H, s, 2CH₂). ¹³C-NMR (DMSO-d₆): 162.5 (CO), 159.7 (CO), 143.3 (4C), 140.1 (4C), 134.8 (2C), 130.5 (4C), 129.8 (2CH), 121.1 (8CH), 119.5 (4CH). C₃₁H₂₈N₈O₂; MW 694.37; Elem. Anal.: Calc C 68.35, H 4.94, N 21.12 Found C 68.00, H 4.55, N 21.35. LC/MS m/z 531 [M + H]+.

**4-(4-((1H-Benz[d][1,2,3]Triazol-1-yl)methyl)(phenoxy)amino)-4-Oxobutanoic Acid (10a)**

Compound 10a was obtained in 10% total yield; m.p. 193–195°C; TLC (chloroform/methanol 9.5/0.5); RF = 0.15; ¹H-NMR (200 MHz, DMSO-d₆): δ: 10.04 (H, s, NH), 8.02 (H, d, J = 8.2, H-4), 7.81 (H, d, J = 8.2, H-7), 7.62-7.50 (3H, m, H-2', 6' and H-5'), 7.40 (H, t, H-6). 7.31 (2H, d, J = 9.2, H-3'), 5.90 (H, s, CH₂), 2.53 (4H, s, 2CH₂). ¹³C-NMR (DMSO-d₆): 173.7 (CO), 170.1 (CO), 145.3 (C), 139.1 (C), 132.5 (C), 130.0 (C), 1284 (2CH), 127.3 (CH), 124.0 (CH), 119.2 (CH), 119.1 (2CH), 110.7 (CH), 50.7 (CH₂), 31.1 (CH₂), 28.7 (CH₂). C₃₁H₁₆N₄O₂; MW 324.33; Elem. Anal.: Calc C 62.95, H 4.97, N 17.27 Found C 62.65, H 4.78, N 17.54. LC/MS m/z 325 [M + H]+.

**N²,N⁶-Bis-(4-(5,6-Dichloro-2H-Benz[d][1,2,3]Triazol-2-yl)phenyl)carbamoyl)picolinic Acid (6f)**

Compound 6f was obtained in 30% total yield; m.p. 240°C; TLC (chloroform/methanol 9/1); RF = 0.1; ¹H-NMR (400 MHz, DMSO-d₆): δ: 11.39 (1H, s, OH), 11.16 (1H, s, NH), 8.80 (1H, s, H-4), 8.58 (1H, t, H-4'), 8.31 (5H, s, H-7, H-2', 3', 5', 6'), 8.16 (1H, d, J = 8.0, H-3'), 7.98 (1H, d, J = 8.0, H-5'). ¹³C-NMR (DMSO-d₆): 164.9 (CO), 161.9 (CO), 148.9 (C), 146.7 (C), 144.7 (C), 140.1 (CH), 138.8 (C), 131.9 (C), 131.5 (C), 131.0 (C), 127.7 (C), 127.2 (CH), 125.8 (CH), 123.5 (CH), 123.4 (CH), 121.8 (CH), 121.6 (CH), 120.9 (CH), 113.0 (CH). C₃₁H₂₈C₄N₄O₄; MW 648.22; Elem. Anal.: Calc C 65.49, H 2.59, N 16.56 Found C 65.39, H 2.72, N 16.64. LC/MS m/z: 429 [M + H]+, 428 [M + H]+.

**N²,N⁶-Bis-(4-(5,6-Dichloro-2H-Benz[d][1,2,3]Triazol-2-yl)phenyl)thiophene-2,5-dicarboxamide (5g)**

Compound 5g was obtained in 11% total yield; m.p. >300°C; TLC (chloroform/methanol 9/1); RF = 0.74; ¹H-NMR (400 MHz, DMSO-d₆): δ: 10.73 (2H, s, NH), 8.51 (4H, s, 2H-4', 2H-7'). 8.32 (4H, d, J = 8.8, 2H-2', 6'), 8.06 (6H, m, 2H-3', 5', H-3', 4'). ¹³C-NMR (DMSO-d₆): 162.5 (CO), 159.7 (CO), 143.3 (4C), 140.1 (4C), 134.8 (2C), 130.5 (4C), 129.8 (2CH), 121.1 (8CH), 119.5 (4CH). C₃₁H₂₈C₄N₄O₂; MW 694.37; Elem. Anal.: Calc C 65.49, H 2.32, N 16.14 Found C 52.00, H 2.55, N 15.90. LC/MS m/z: 695 [M + H]+, 697 [M + H]+.

**5'-((4-(1H-Benz[d][1,2,3]Triazol-1-yl)methyl)phenyl)aminoo)-5-Oxopentanoic Acid (10b)**

Compound 10b was obtained in 25% total yield; m.p. 152–154°C; TLC (chloroform/methanol 9.5/0.5); RF = 0.20; ¹H-NMR (200 MHz, DMSO-d₆): δ: 9.99 (2H, s, 2NH), 8.04 (1H, d, J = 7.6, H-4), 7.82 (1H, d, J = 8.0, H-7), 7.56-7.42 (3H, m, H-2', 6', H-5), 7.38 (1H, t, H-6). 7.28 (2H, d, J = 8.4, H-3', 5'), 5.90 (2H, s, CH₂), 2.31 (2H, t, CH₂CO), 2.22 (2H, t, CH₂CO), 1.77 (2H, m, CH₂CH₂CH₂). ¹³C-NMR (DMSO-d₆): 174.5 (CO), 170.9 (CO), 145.3 (C), 139.1 (C), 132.5 (C), 130.1 (C), 128.6 (2CH), 128.3 (CH), 124.0 (CH), 119.2 (CH), 119.1 (2CH), 110.7 (CH), 50.7 (CH₂), 35.4 (2CH₂), 20.5 (CH₂). C₃₁H₂₈N₄O₂; MW 544.61; Elem. Anal.: Calc C 63.87, H 5.18, N 20.58 Found C 63.64, H 5.18, N 20.80. LC/MS m/z: 545 [M + H]+.
6-((1H-Benzol[d][1,2,3]triazol-1-yl) methyl)phenylamino)-6-oxohexanoic Acid (10c)

Compound 10c was obtained in 33% total yield; m.p. 134–136 °C; TLC (chloroform/methanol 9.5/0.5); Rf = 0.23; 1H-NMR (200 MHz, DMSO-d6) δ: 9.91 (2H, s, 2NH), 8.03 (2H, d, J = 8.0, 2H-4), 7.82 (2H, d, J = 8.2, 2H-7), 7.58–7.41 (6H, m, H-2′,5′, 2H-5), 7.38 (2H, t, 2H-7), 7.27 (4H, d, J = 8.6, 2H-3′,5′), 5.90 (4H, s, 2CH2), 2.27 (4H, t, 2CH2CO), 1.54 (4H, m, CH2CH2). 13C-NMR (DMSO-d6): δ: 171.6 (2CO), 145.8 (2C), 139.6 (2C), 133.0 (2C), 130.6 (2C), 128.4 (2CH), 124.4 (2CH), 119.7 (2CH), 119.6 (4CH), 111.2 (2CH), 51.2 (2CH2), 36.6 (2CH3), 25.2 (2CH3). C30H24N8O2; MW 558.63; Elem. Anal.: Calcd C 64.76, H 5.72, N 15.90 Found C 64.33, H 5.54, N 22.00.

N1,N4′-Bis(4-((1H-Benzol[d][1,2,3]triazol-1-yl)methyl)phenyl)Fumaramide (7d)

Compound 7d was obtained in 12% total yield; m.p.: 266–269 °C; TLC (chloroform/methanol 9.5/0.5); Rf = 0.41; 1H-NMR (200 MHz, DMSO-d6) δ: 10.58 (2H, s, 2NH), 8.04 (2H, d, J = 8.2, 2H-4), 7.85 (2H, d, J = 8.2, 2H-7), 7.76 (4H, d, J = 8.4, 2H-2′,5′), 7.53 (2H, t, 2H-5) 7.43 (2H, t, 2H-6) 7.34 (4H, d, J = 8.4, 2H-3′,5′), 7.14 (2H, s, CH = CH), 5.94 (4H, s, 2CH2). 13C-NMR (DMSO-d6): δ: 162.1 (2CO), 143.3 (2C), 138.6 (2C), 134.0 (2CH), 132.5 (2C), 131.1 (2CH), 128.4 (2CH), 127.4 (2CH), 124.0 (2CH), 119.6 (2CH), 119.5 (2CH), 110.7 (2CH), 50.7 (2CH2). C30H24N8O2; MW 528.56; Elem. Anal.: Calcd C 64.76, H 5.72, N 15.90 Found C 65.00, H 5.93, N 16.00. LC/MS: m/z 353 [M + H]+.

(3E)-4-((4-((1H-Benzol[d][1,2,3]triazol-1-yl)methyl)phenylamino)-4-oxobut-2-enoic Acid (10d)

Compound 10d was obtained in 30% total yield; m.p. 290–293 °C; TLC (chloroform/methanol 9.5/0.5); Rf = 0.11; 1H-NMR (200 MHz, DMSO-d6) δ: 10.22 (1H, s, NH), 8.04 (1H, d, J = 7.6, 2H-4), 7.82 (1H, d, J = 9.0, H-7), 7.63 (2H, d, J = 8.2, H-2′,5′), 7.52 (1H, t, H-5) 7.42 (1H, t, H-6) 7.30 (2H, d, J = 8.2, H-3′,5′), 6.61 (2H, s, CH = CH), 5.91 (2H, s, CH2). 13C-NMR (DMSO-d6): δ: 168.1 (CO), 164.0 (CO), 145.4 (C), 139.1 (C), 134.0 (2CH), 132.5 (C), 130.5 (C), 130.0 (CH), 128.4 (2CH), 127.3 (CH), 124.0 (CH), 119.6 (CH), 119.4 (CH), 119.2 (CH), 110.7 (CH), 50.7 (CH2). C32H19N4O4; MW 572.32; Elem. Anal.: Calcd C 68.38, H 4.35, N 21.75 Found C 68.40, H 4.35, N 22.00. LC/MS: m/z 580 [M + H]+.
6-4-{((1H-Benzo[d][1,2,3]Triazol-1-yl)methyl}phenylcarbamoyl)Picolinic Acid (10f)

Compound 10f was obtained in 30% total yield; m.p. 230–232 °C; TLC (chloroform/methanol 9.5/0.5); Rf = 0.10; 1H-NMR (200 MHz, DMSO-d6) δ: 11.00 (H, s, NH), 8.40–8.32 (3H, m, H-3′,4′,6′), 8.06 (2H, d, J = 8.2, 2H arom), 7.86 (2H, d, J = 7.8, H-2′,6′), 7.57–7.50 (2H, m, 2H arom), 7.42 (H, d, J = 7.8, H-3′,5′), 5.99 (2H, s, CH2). 13C-NMR (DMSO-d6) δ: 166.8 (CO), 162.8 (CO), 153.2 (C), 148.9 (C), 145.4 (C), 138.4 (C), 132.6 (C), 127.4 (2CH), 124.0 (2CH), 123.2 (CH), 120.6 (2CH), 119.2 (2CH), 110.7 (2CH), 50.7 (CH2). C34H35N8O2; MW 586.69; Elem. Anal.: Calcd C 65.44, H 4.24, N 19.32 Found C 65.33, H 4.35, N 19.03. LC/MS: m/z 587 [M + H]+.

N2,N5-Bis(4-{(1H-Benzo[d][1,2,3]Triazol-1-yl)methyl}phenyl)phenyl) Thiophene-2,5-Dicarboxamide (7g)

Compound 7g was obtained in 20% total yield; m.p. >300 °C; TLC (chloroform/methanol 9.5/0.5); Rf = 0.31; 1H-NMR (200 MHz, DMSO-d6) δ: 10.27 (2H, s, 2NH), 8.15–8.07 (2H, d, J = 8.4, H-3′,4′), 7.70–7.67 (4H, m, 4Harom), 7.64 (4H, d, J = 8.2, 2H-2′,6′), 7.46 (2H, t, J = 7.2, 2H arom), 7.32 (2H, t, J = 7.2, 2H arom), 7.70 (4H, d, J = 8.2, 2H-3′,5′), 5.94 (4H, s, 2CH2). 13C-NMR (DMSO-d6) δ: 160.1 (2CO), 145.3 (2C), 140.9 (2C), 138.6 (2C), 132.6 (2C), 130.9 (2C, 129.5 (2CH), 128.3 (4CH), 127.4 (2CH), 124.0 (2CH), 120.6 (2CH), 120.5 (2CH), 119.2 (2CH), 110.7 (2CH), 50.7 (2CH2). C35H36N8O2S; MW 584.65; Elem. Anal.: Calcd C 65.74, H 4.14, N 19.17 Found C 65.44, H 4.24, N 19.32 LC/MS: m/z 585 [M + H]+.

5-{4-{((1H-Benzo[d][1,2,3]Triazol-1-yl)methyl}phenyl}phenyl) thiophene-2-Carbonylic Acid (10g)

Compound 10g was obtained in 25% total yield; m.p. >300 °C; TLC (chloroform/methanol 9.5/0.5); Rf = 0.10; 1H-NMR (200 MHz, DMSO-d6) δ: 10.27 (H, s, NH), 8.06–8.04 (2H, d, J = 8.4, H-3′,4′), 7.85 (2H, d, J = 8.2, 2H-2′,6′), 7.70 (4H, d, J = 8.2, 2H-3′,5′), 7.52 (H, t, J = 7.2, H arom), 7.40 (H, t, J = 7.2, H arom), 7.35 (2H, d, J = 8.2, H arom); 5.94 (2H, s, CH2). 13C-NMR (DMSO-d6) δ: 160.1 (2CO), 145.3 (2C), 140.9 (2C), 138.6 (2C), 132.6 (2C), 130.9 (2C), 129.5 (2CH), 128.3 (4CH), 127.4 (2CH), 124.0 (2CH), 120.6 (2CH), 120.5 (2CH), 119.2 (2CH), 110.7 (2CH), 50.7 (2CH2). C35H36N8O2S; MW 578.40; Elem. Anal.: Calcd C 60.31, H 3.73, N 14.81 Found C 60.05, H 3.71, N 14.65 LC/MS: m/z 379 [M + H]+.

N1,N5-Bis(4-{((5,6-Dimethyl-1H-Benzo[d][1,2,3]Triazol-1-yl)methyl}phenyl) Glutaramide (8b)

Compound 8b was obtained in 18% total yield; m.p. 253–255 °C; TLC (chloroform/methanol 9.8/0.2); Rf = 0.54; 1H-NMR (200 MHz, DMSO-d6) δ: 9.95 (2H, s, 2NH), 7.79 (2H, s, H-3′,4′), 7.65 (2H, s, H-7 e H-2′,6′), 7.60 (4H, d, J = 8.6, H-3′,5′), 7.28 (4H, d, J = 8.6, 2H-3′,5′), 5.81 (4H, s, 2CH2), 2.42 (4H, t, 2CH2CO), 2.35 (12H, s, 4CH3), 1.86 (2H, m, CH2). 13C-NMR (DMSO-d6) δ: 171.7 (2CO), 144.0 (2C), 139.6 (2CH), 137.8 (2C), 133.9 (2CH), 132.0 (2C), 130.7 (2C), 128.6 (4CH), 119.7 (2CH), 119.6 (2CH), 118.5 (2CH), 109.6 (2CH), 51.0 (2CH2), 36.3 (2CH2), 21.7 (CH2), 20.7 (2CH3), 20.3 (2CH3). C23H28N6O2; MW 600.71; Elem. Anal.: Calcd C 69.98, H 6.04, N 18.65 Found C 70.00, H 6.21, N 18.70. LC/MS: m/z 601 [M + H]+.

5-{4-{((5,6-Dimethyl-1H-Benzo[d][1,2,3]Triazol-1-yl)methyl}phenyl) amino)-5-Oxopentanoic Acid (11b)

Compound 11b was obtained in 25% total yield; m.p. 270–272 °C; TLC (chloroform/methanol 9.8/0.2); Rf = 0.35; 1H-NMR (200 MHz, DMSO-d6) δ: 10.35 (1H, s, NH), 7.78 (1H, s, H-4), 7.55 (3H, d, J = 8.6, H-7 e H-2′,6′), 7.23 (2H, d, J = 8.4, H-3′,5′), 5.80 (2H, s, CH2), 2.34 (6H, s, 2CH3), 2.02 (4H, t, 2CH2CO), 1.72 (2H, m, CH2). 13C-NMR (DMSO-d6) δ: 174.5 (CO), 170.9 (CO), 145.3 (C), 139.1 (C), 136.8 (2CH) 132.5 (C), 130.1 (C), 128.6 (2CH), 124.3 (2CH), 122.0 (2CH), 50.7 (CH3), 35.4 (2CH2), 20.5 (CH). C35H36N8O2; MW 566.41; Elem. Anal.: Calcd C 65.56, H 6.05, N 15.29 Found C 70.00, H 6.21, N 18.70. LC/MS: m/z 367 [M + H]+.
**N¹,N⁶-Bis(4-((5,6-Dimethyl-1H-Benzo[d][1,2,3]Triazol-1-yl)methyl)phenyl) Adipamide (8c)**

Compound 8c was obtained in 23% total yield; m.p. 283–285°C; TLC (chloroform/methanol 9.8/0.2); Rf = 0.30; ¹H-NMR (200 MHz, DMSO-d₆) δ: 9.90 (2H, s, 2NH), 7.78 (2H, s, 2H-4), 7.57 (2H, s 2H-7), 7.52 (4H, d, J = 8.6, H-2′,6′), 7.23 (4H, d, J = 8.6, 2H-3′,5′), 5.81 (4H, s, 2CH₂), 2.34 (12H, s, 4CH₃), 2.25 (4H, t, 2CH₂CO), 1.56 (4H, t, 2CH₂). ¹³C-NMR (DMSO-d₆) δ: 171.1 (2CO), 144.6 (2C), 139.0 (2C), 137.3 (2C), 133.4 (2C), 131.6 (2C), 130.3 (2C), 128.1 (4CH), 119.2 (4CH), 118.1 (2CH), 109.7 (2CH), 50.5 (2CH₂), 36.2 (2CH₂), 28.6 (2CH₂), 20.3 (2CH₃), 19.8 (2CH₃). C₃₈H₃₅N₉O₂; MW 635.72; Elem. Anal.: Calcd C 69.90, H 5.23, N 15.99 Found C 69.85, H 5.52, N 19.17. LC/MS: m/z 401 [M + H]+.

**N¹,N⁴-Bis(4-((5,6-Dimethyl-1H-Benzo[d][1,2,3]Triazol-1-yl)methyl)phenyl) amino)-4-Oxobut-2-Enoic Acid (11d)**

Compound 11d was obtained in 20% total yield; m.p. 256–258°C; TLC (chloroform/methanol 8.5/1.5); Rf = 0.24; ¹H-NMR (200 MHz, DMSO-d₆) δ: 10.29 (1H, s, NH), 7.78 (1H, s, H-4), 7.65 (1H, s, H-7), 7.60 (2H, d, J = 8.6, H-2′,6′), 7.25 (2H, d, J = 8.6, H-3′,5′), 6.65 (2H, s, CH=CH), 5.82 (2H, s, CH₂), 2.34 (6H, s, 2CH₂). ¹³C-NMR (DMSO-d₆) δ: 169.2 (CO), 164.1 (CO), 144.6 (C), 139.1 (C), 137.3 (C), 133.4 (CH), 131.6 (C), 128.3 (CH), 128.2 (CH), 119.4 (2CH), 118.1 (2CH), 109.7 (2CH), 50.5 (CH₂), 20.3 (CH₃), 19.8 (CH₃). C₇₃H₇₅N₉O₂; MW 350.37; Elem. Anal.: Calcd C 66.13, H 5.18, N 15.99 Found C 66.15, H 5.23, N 15.78. LC/MS: m/z 351 [M + H]+.

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Ibba et al. Synthesis Antitumor Antiviral Activities Benzotriazoledicarboxamide Frontiers in Chemistry | www.frontiersin.org May 2021 | Volume 9 | Article 660424
6-((4-(5,6-Dimethyl-1H-Benzod][1,2,3]
Triazol-1-yl)methyl)phenyl)carbamoyl)
Picolinic Acid (11f)

Compound 11f was obtained in 27% total yield; m.p. 214–216°C; TLC (chloroform/methanol 8.5/1.5); Rₚ = 0.23; ¹H-NMR (200 MHz, DMSO-d₆) δ: 11.41 (1H, s, NH), 8.22–8.01 (3H, m, H-3',6',4'), 7.87 (2H, d, J = 8.6, H-2',6') 7.79 (1H, s, H-4), 7.62 (1H, s, H-1'), 7.32 (2H, d, J = 8.6, H-3',5'), 5.87 (2H, s, CH₂), 2.34 (6H, s, 2CH₃). ¹³C-NMR (DMSO-d₆) δ: 164.7 (CO), 161.6 (CO), 150.0 (2C), 148.2 (2C), 144.3 (C), 140.1 (CH), 138.0 (C), 132.0 (C), 131.2 (C), 130.8 (C), 128.6 (CH), 128.5 (CH), 127.0 (CH), 125.8 (CH), 121.3 (CH), 120.9 (CH), 120.6 (CH), 112.5 (CH), 50.8 (CH₂). C₂₂H₂₄N₄O₃S; MW 401.42; Elem. Anal.: Calcd C 65.83, H 4.77, N 17.45 Found C 66.10, H 4.80, N 17.55. LC/MS: m/z 402 [M + H]⁺.

N²,N²-Bis((4-(5,6-Dimethyl-1H-Benzod][1,2,3]Triazol-1-yl)methyl)phenyl)
Thiophene-2,5-Dicarboxamide (8g)

Compound 8g was obtained in 10% total yield; m.p. 225–227°C; TLC (chloroform/methanol 9.8/0.2); Rₚ = 0.47; ¹H-NMR (200 MHz, DMSO-d₆) δ: 10.52 (2H, s, 2NH), 8.02 (2H, s, H-3',6'), 7.79 (2H, s, 2H-4), 7.49 (4H, d, J = 7.8, 2H-2',6'), 7.61 (2H, s, 2H-7), 7.31 (4H, d, J = 7.8, 2H-3',5'), 5.87 (4H, s, 2CH₂), 2.35 (12H, s, 4CH₃). ¹³C-NMR (DMSO-d₆) δ: 161.5 (2CO), 145.7 (2C), 144.6 (2C), 143.8 (2C), 137.9 (2C), 137.3 (2C), 136.3 (2C), 133.9 (2CH), 131.7 (2C), 131.5 (2C), 128.5 (2CH), 128.4 (2CH), 120.7 (2CH), 120.6 (2CH), 119.3 (2CH), 118.1 (2CH), 109.7 (2CH), 52.6 (2CH₂), 20.4 (2CH₃), 20.3 (2CH₃). C₃₆H₃₂N₈O₂S; MW 640.76; Elem. Anal.: Calcd C 60.81, H 4.75, N 17.49 Found C 60.50, H 4.80, N 17.14. LC/MS: m/z 641 [M + H]⁺.

5-((4-((5,6-Dimethyl-1H-Benzod][1,2,3]
Triazol-1-yl)methyl)phenyl)carbamoyl)
thiophene-2-carboxylic Acid (11g)

Compound 11g was obtained in 40% total yield; m.p. >300°C; TLC (chloroform/methanol 8.5/1.5); Rₚ = 0.21; ¹H-NMR (200 MHz, DMSO-d₆) δ: 10.22 (2H, s, NH), 7.79 (2H, d, J = 3.8, H-3',6'), 7.79 (1H, s, H-4), 7.68 (2H, d, J = 8.0, H-2',6'), 7.60 (1H, s, H-7), 7.29 (2H, d, J = 8.0, H-3',5'), 5.86 (2H, s, CH₂), 2.34 (6H, s, 2CH₃). ¹³C-NMR (DMSO-d₆) δ: 165.2 (CO), 160.2 (CO), 144.6 (C), 138.5 (C), 137.4 (2C), 133.5 (2C), 131.6 (1C), 131.1 (C), 128.1 (2CH), 120.5 (2CH), 118.1 (2CH), 109.7 (2CH), 50.4 (CH₂), 20.4 (CH₃), 19.8 (CH₃). C₁₇H₁₄Cl₂N₄O₃; MW 393.22; Elem. Anal.: Calcd C 53.91, H 3.32, N 16.77 Found C 53.65, H 3.46, N 16.45. LC/MS: m/z 394 [M + H]⁺, 393 [M + H]⁺.

N²,N²-Bis((4-((5,6-Dichloro-1H-Benzod][1,2,3]Triazol-1-yl)methyl)phenyl)
Glutaramide (9b)

Compound 9b was obtained in 10% total yield; m.p. 204–206°C; TLC (chloroform/methanol 9.5/0.5); Rₚ = 0.70; ¹H-NMR (400 MHz, DMSO-d₆) δ: 9.94 (2H, s, NH), 8.48 (2H, s, H-4'), 8.37 (2H, s, H-7), 7.56 (4H, d, J = 8.0, H-3',5'), 7.31 (4H, d, J = 8.0, H-2',6'), 5.90 (4H, s, CH₂-N), 2.51 (4H, s, CH₂CH₂). C₂₃H₁₈Cl₂N₂O₂; MW 416.93; Elem. Anal.: Calcd C 45.95, H 3.47, N 14.79 Found C 45.67, H 3.39, N 14.36. LC/MS: m/z 417 [M + Na]⁺, 415 [M + Na]⁺, 393 [M + H]⁺.

5-((4-((5,6-Dichloro-1H-Benzod][1,2,3]
Triazol-1-yl)methyl)phenyl)amino)-5-Oxopentanoic Acid (12b)

Compound 12b was obtained in 15% total yield; m.p. 173–175°C; TLC (chloroform/methanol 9.5/0.05); Rₚ = 0.35; ¹H-NMR (400 MHz, DMSO-d₆) δ: 10.30 (1H, s, NH), 8.47 (1H, s, H-4), 8.36 (1H, s, H-7), 7.57 (2H, d, J = 8.0, H-3',5'), 7.30 (2H, d, J = 8.0, H-2',6'), 5.89 (2H, s, CH₂-N), 2.29 (2H, t, CH₂-CO-N), 2.05–2.01 (2H, m, CH₃-COOH), 1.74 (2H, t, CH₂-CO-N). ¹³C-NMR (DMSO-d₆) δ: 171.4 (2CO), 144.3 (C), 139.4 (C), 131.9 (C), 130.7 (C), 129.5 (C), 128.5 (2CH), 127.2 (C), 120.5 (CH), 119.2 (2CH), 112.5 (CH), 50.9 (CH₂-N), 36.0 (CH₂), 35.9 (CH₃), 21.5 (CH₃). C₁₈H₁₆Cl₂N₂O₂; MW 407.25; Elem. Anal.: Calcd C 52.09, H 3.96, N 13.76 Found C 52.86, H 4.16, N 14.14. LC/MS: m/z 447 [M + K]⁺, 445 [M + K]⁺.
N⁷,N⁶-bis(4-((5,6-Dichloro-1H-Benzo[d][1,2,3]Triazol-1-yl)methyl)phenyl) Adipamide (9c)

Compound 9c was obtained in 15% total yield; m.p. >300°C; TLC (chloroform/methanol 9.5/0.5); Rf = 0.63; 1H-NMR (400 MHz, DMSO-d₆) δ: 9.92 (2H, s, NH), 8.48 (2H, s, H-4), 8.36 (2H, s, H-7), 7.55 (4H, d, J = 8.0, H-3′,5′), 7.31 (4H, d, J = 8.0, H-2′,6′), 5.90 (4H, s, CH₂-N), 2.29 (4H, s, 2CH₂CO), 1.58 (4H, s, CH₂-CH₂). 13C-NMR (DMSO-d₆) δ: 171.1 (2C), 144.3 (2C), 139.3 (2C), 131.9 (2C), 130.7 (2C), 129.6 (2C), 128.5 (4CH), 127.2 (2C), 120.6 (2CH), 119.2 (4CH), 112.5 (2CH), 50.9 (2CH₂-N), 36.2 (2CH₂), 24.7 (2CH₂). C₃₂H₂₅Cl₂N₄O₂; MW 696.41; Elem. Anal.: Calc'd C 55.19, H 3.76, N 16.09 Found C 55.10, H 3.80, N 15.89. LC/MS: m/z 697 [M + H⁺], 695 [M + H⁺].

6-((4-((5,6-Dichloro-1H-Benzo[d][1,2,3]Triazol-1-yl)methyl)phenyl)amino)-6-Oxohexanoic Acid (12c)

Compound 12c was obtained in 13% total yield; m.p. 228–230°C; TLC (chloroform/methanol 95.0/5.5); Rf = 0.38; 1H-NMR (400 MHz, DMSO-d₆) δ: 10.05 (1H, s, NH), 8.48 (1H, s, H-4), 8.37 (1H, s, H-7), 7.57 (2H, d, J = 8.0, H-3′,5′), 7.31 (2H, d, J = 8.0, H-2′,6′), 5.90 (2H, s, CH₂-N), 2.27 (2H, t, CH₂-CO), 2.12 (2H, t, CH₂-CH₂), 1.58–1.49 (4H, m, CH₂-CH₂). 13C-NMR (DMSO-d₆) δ: 171.3 (2CO), 144.3 (C), 139.3 (C), 131.9 (C), 130.7 (C), 129.6 (C), 128.5 (2CH₂), 127.2 (C), 120.6 (CH), 119.2 (2CH₂), 112.5 (CH), 50.9 (CH₂-N), 36.1 (2CH₂), 24.9 (CH₂), 24.6 (CH₂). C₁₉H₁₅Cl₂N₄O₂; MW 421.28; Elem. Anal.: Calc'd C 54.17, H 4.31, N 13.30 Found C 53.85, H 4.30, N 13.62. LC/MS: m/z 445 [M + Na⁺], 443 [M + Na⁺].

N⁷,N⁶-Bis(4-((5,6-Dichloro-1H-Benzo[d][1,2,3]Triazol-1-yl)methyl)phenyl) Pyridine-2,6-Dicarboxamide (9f)

Compound 9f was obtained in 13% total yield; m.p. >300°C; TLC (chloroform/methanol 9/1); Rf = 0.84; 1H-NMR (400 MHz, DMSO-d₆) δ: 10.91 (2H, s, NH), 8.50 (2H, s, H-2,4), 8.43 (2H, s, 2H-7), 8.40–8.36 (1H, m, H-4″), 8.32–8.25 (2H, m, H-3″,5″), 7.82 (4H, d, J = 8.4, H-2′-6′), 7.45 (4H, d, J = 8.4, H-2′-6′), 5.99 (4H, s, 2CH₂). 13C-NMR (DMSO-d₆) δ: 162.0 (2CO), 148.6 (2C), 144.7 (2C), 143.4 (2C), 139.9 (2C), 138.9 (2C), 132.0 (2C), 131.6 (2C), 128.6 (1CH₂), 123.6 (4CH), 121.9 (4CH), 121.2 (1CH), 120.9 (2CH), 120.6 (2CH), 50.9 (2CH₂). C₁₉H₁₅Cl₂N₄O₂; MW 717.39; Elem. Anal.: Calc'd C 55.25, H 2.95, N 17.57 Found C 55.00, H 3.10, N 17.78. LC/MS: m/z 717 [M + K⁺], 715 [M + H⁺].

6-((4-((5,6-Dichloro-1H-Benzo[d][1,2,3]Triazol-1-yl)methyl)phenyl)carbamoyl) Picolinic Acid (12f)

Compound 12f was obtained in 40% total yield; m.p. 175–177°C; TLC (chloroform/methanol 9/1); Rf = 0.16; 1H-NMR (400 MHz, DMSO-d₆) δ: 11.06 (1H, s, OH), 10.96 (1H, s, NH), 8.52 (1H, s, H-4), 8.45–8.41 (3H, t), 8.4–8.31 (1H, m, H-3″,5″), 7.94 (1H, d, J = 8.4, H-3″), 7.85 (1H, d, J = 8.4, H-5″), 7.50 (2H, d, J = 8.4, H-2′-6′), 6.03 (2H, s, CH₂). 13C-NMR (DMSO-d₆) δ: 164.7 (CO), 161.6 (CO), 149.0 (2C), 146.2 (C), 144.3 (C), 140.1 (CH), 138.0 (C), 132.0 (C), 131.2 (C), 130.8 (C), 128.6 (CH), 128.5 (CH), 127.0 (CH), 125.8 (CH), 121.3 (CH), 120.9 (CH), 120.6 (CH), 112.5 (CH), 50.8 (CH₂). C₁₉H₁₅Cl₂N₄O₂; MW 442.25; Elem. Anal.: Calc'd C 54.32, H 2.96, N 15.84 Found C 54.32, H 3.06, N 15.74. LC/MS: m/z 443 [M + K⁺], 441 [M + H⁺].
SCHEME 1 | Synthesis of N,N'-bis-[4-[(1H)-benzo[d][1,2,3]triazol-1(2)yl]phenyl]alkyl(aryl) dicarboxamides (3a-g; 5e-g) and 4-[(4-(5,6-dichloro-1H)-benzo[d][1,2,3]triazol-1(2)-yl]phenyl]amino]-4-oxoalkyl(aryl)oxic acids (4a-d,f; 6f). 1) DMF, TEA, r.t., 3–72 h.

SCHEME 2 | Synthesis of N,N'-bis[4-((5,6-R-1H-benzo[d][1,2,3]triazol-1-yl)methyl)phenyl]alkyl(aryl) dicarboxamides (7a-g; 8a-g; 9a-g) and 4-[(4-((5,6-R-1H-benzo[d][1,2,3]triazol-1-yl)methyl)phenyl)amino]-4-oxoalkyl(aryl)oxic acids (10a-g; 11a-g; 12a-d,f). 1) DMF, TEA, r.t., 3–72 h.

SCHEME 3 | Synthesis of 4-((5,6-R-1H-benzo[d][1,2,3]triazol-1-yl)methyl)aniline (16–18). 1) Cs₂CO₃, DMF, 60°C for 48 h; 2) NH₂NH₂ (1:20), Pd/C (10% w/w), EtOH, 80°C for 2 h (to afford compounds 16 and 17); 3) CH₃NHNH₂ (1:10), EtOH, 100°C for 48 h in autoclave (to afford compound 18).
FIGURE 3] Synthesized derivatives, N,N'-bis[4-H(2H)-benzo[1,2,3]triazol-1 (2)-yl]phenyl alkyl (aryl)dicarboxamides (series 3 and 5) and N,N'-bis[4-H-benzo [1,2,3]triazol-1-yl) (methyl)phenyl alkyl (aryl)dicarboxamides (series 7–9), 4- (4-(5,6-dichloro-1-H-benzo[1,2,3]triazol-1 (2)-yl)phenyl)amino)-4-oxoalkyl (aryl)ic acids (series 4 and 6), 4- (4-((5,6-R-1-H-benzo[d][1,2,3]triazol-1-yl)methyl)phenyl)amino)-4-oxoalkyl (aryl)ic acids (series 10–12).

TABLE 1 | Antiviral activity and cytotoxicity results for compounds 9a, 9b among the bis-benzotriazole-dicarboxamides and 4a-d among the mono-substituted acidic derivatives.

| Dicarboxamides | Cytotoxicity in Vero-76 cell-line | Efficacy in plaque reduction assay | Mono-substituted Cytotoxicity in Vero-76 cell-line | Efficacy in plaque reduction assay |
|---------------|---------------------------------|----------------------------------|---------------------------------|----------------------------------|
|               | CC50 (µM)a                        | EC50 (µM)b                       | CC50 (µM)a                        | EC50 (µM)b                       |
| 9a            | >100                             | 23                               | 90                              | 13                               |
| 9b            | 75                               | 52                               | >75 4a                           | >90 13                           |
| P             | >100                             | 0.005                            | >100 4a                          | 45 44                            |
| 2ec           | >100                             | 23                               | >100 4a                          | 0.005 23                         |

Pleconaril (P) and 2‘-C-ethynyl-cytidine (2ec) were used as control. Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%.

aCompound concentration (µM) required to reduce the viability of mock-infected Vero-76 cells by 50%, as determined by the MTT method.
bCompound concentration (µM) required to reduce the plaque number of CV-B5 or Sb-1 by 50% in Vero-76 monolayers.
TABLE 2 | Anti-proliferative activity for compounds 3b, 3d, 4d, and 9b against human leukemia-lymphoma-derived cell lines and solid tumor-derived cell-lines.

| Compounds      | CCRF-CEM<sup>a</sup> | WIL-2NS<sup>a</sup> | CCRF-SB<sup>a</sup> | SK-MEL28<sup>a</sup> | SK-MES<sup>a</sup> | DU145<sup>a</sup> | HeLa<sup>a</sup> | CRL 7065<sup>a</sup> |
|----------------|----------------------|---------------------|---------------------|----------------------|-------------------|-----------------|-----------------|-------------------|
| 3b             | 0.5 ± 0.1            | >100                | 0.35 ± 0.07         | 6.2 ± 1.3            | 6.8 ± 3.5         | 5.1 ± 0         | 7.1 ± 2.7       | 0.75 ± 0.05       |
| 3d             | 0.07 ± 0.02          | >100                | 7.9 ± 0.9           | >100                 | >100              | >100            | >100            | 0.37 ± 0.04       |
| 4d             | 3.3 ± 1.7            | 64 ± 10             | 0.51 ± 0.13         | >100                 | >100              | >100            | >100            | 1.7 ± 0.1         |
| 9b             | 5.5 ± 1.8            | 23 ± 7              | 8.5 ± 3.2           | 2.6±0.7              | 9.9±3.1           | 5.2 ± 0.4       | 5.4 ± 0.7       | 6.7 ± 2.4         |
| Vincristine    | 0.003 ± 0.0001       | 0.006 ± 0.002       | 0.003 ± 0.001       | >4                   | 0.01±0.02         | 0.02 ± 0.01     | 0.05 ± 0.02     | >4                |
| Doxorubicin    | 0.02±0.01            | 0.06±0.01           | 0.03±0.02           | 1.1±0.2              | 0.65±0.05         | 0.3 ± 0.1       | 1.8 ± 0.2       | 2.0 ± 0.2         |

Control is represented by CRL 7065 cell line. Vincristine and Doxorubicin were used as positive controls.

<sup>a</sup>Compound concentration required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. Data represent mean values (±SD) for three independent determinations.

<sup>b</sup>Human acute T-lymphoblastic leukemia.

<sup>c</sup>Human splenic B-lymphoblastoid cells.

<sup>d</sup>Human acute B-lymphoblastic.

<sup>e</sup>Human skin melanoma.

<sup>f</sup>Human lung squamous carcinoma.

<sup>g</sup>Human prostate carcinoma.

<sup>h</sup>Human cervix carcinoma.

<sup>i</sup>Human foreskin fibroblasts.

**N²,N²-Bis(4-((5,6-Dichloro-1H-Benzo[d][1,2,3]Triazol-1-yl)methyl)phenyl) Thiophene-2,5-Dicarboxamide (9g)**

Compound 9g was obtained in 11% total yield; m.p. >300°C; TLC (chloroform/methanol 9:1); Rf = 0.82;<sup>2</sup> 1H-NMR (400 MHz, DMSO-d<sub>6</sub>): δ = 10.43 (2H, s, NH), 8.49 (2H, s, 2H-4), 8.40 (2H, s, 2H-3), 8.01 (2H, s, H-3<sup>b</sup>,<sup>c</sup>), 7.71 (4H, d, J = 8.4, 2H-3<sup>d</sup>,<sup>e</sup>), 7.39 (4H, d, J = 8.4, 2H-2<sup>c</sup>), 5.96 (4H, s, 2CH2). 13C-NMR (DMSO-d<sub>6</sub>): δ = 159.3 (2CO), 144.3 (2C), 143.9 (2C), 138.4 (2C), 134.1 (2C), 132.0 (2C), 130.9 (2C), 130.8 (2CH2), 129.4 (4CH), 128.5 (2CH2), 127.2 (2C), 120.7 (4CH), 112.5 (2CH2), 50.8 (2CH2). C<sub>42</sub>H<sub>20</sub>Cl<sub>4</sub>N<sub>8</sub>O<sub>2</sub>S; MW 722.43; Elem. Anal.: Calcd C 52.88, H 3.00, N 15.67. LC/MS: m/z 761 [M + K]<sup>+</sup>, 721 [M + H]<sup>+</sup>.

**RESULTS AND DISCUSSION**

**Chemistry**

Synthetic pathways constructed to obtain the desired products are illustrated in Scheme 1 and Scheme 2, while Scheme 3 shows the synthetic route to synthesize the benzyl-intermediates. 4-((5,6-Dichloro-1(2H)-benzo[d][1,2,3]triazol-1-yl)aniline intermediates (13 and 15) were synthesized according to the previously described procedure (Carta et al., 2007). Diaryl-dichloride (14a-g) and 1-(chloromethyl)-4-nitrobenzene reagents were commercially purchased (Sigma-Aldrich). All the designed bis-benzotriazole-dicarboxamides derivatives were prepared by condensation of the corresponding benzotriazole-anilines (13,15 and 16–18) with the suitable diaryl dichloride (14a-g) in N,N-dimethylformamide (DMF) in the presence of triethylamine (TEA). These synthetic routes afforded the desired compounds bis-benzotriazole-dicarboxamides (3a-g, 5e-g, 7a-g, 8a-g, 9a-g) and the corresponding mono-substituted acidic derivatives (4a-d,f, 6f, 10a-g, 11a-g, 12a-d,f) as secondary products, that also were separated, purified and biologically evaluated. The bis-benzotriazole-dicarboxamides and the mono-substituted acidic derivatives were generally obtained in variable ratios. Intermediates 16–18 were obtained as depicted in Scheme 3, by a first substitution of the properly substituted benzotriazole (19–21) with 1-(chloromethyl)-4-nitrobenzene in basic conditions for triethylamine (TEA). The two isomers were separated from the 5,6-(R)-1-(2-(4-nitrobenzyl)-1H-benzo[d][1,2,3]triazole mixture and the 5,6-(R)-1-(4-nitrobenzyl)-1H-benz[d][1,2,3]triazoles (22–24) were subjected to reduction by two different synthetic pathways. Intermediates 16 and 17 were obtained by reduction of 22 and 23 respectively, with hydrazine and Palladium on carbon in ethanol, while compound 18 was gained by reduction of 24 solved in ethanol with methylhydrazine in the autoclave for 48 h.

**Antiviral activity**

All the 56 synthesized compounds, depicted in Figure 3, were tested in a plaque-reduction assay for antiviral activity and in a cell-based assay for cytotoxicity. Viruses selected for the assay were Cox sackievirus B5 (CV-B5), a well-known human pathogen and the Poliovirus strain Sb-1, both belonging to the family of Picornaviridae. Compounds cytotoxicity was evaluated against three cell lines (Vero-76, MDBK, BHK-21). Table 1 shows the antiviral activity results and the corresponding cytotoxicity against the cell-line that supports viral replication, Vero-76. Results for the only active compounds are reported: derivatives 9a, 9b among the bis-benzotriazole-dicarboxamides and 4a-d among the mono-substituted acidic derivatives. The remaining derivatives results are not reported in the table since they turned out not considerably active (EC<sub>50</sub> values exceeded 100 µM). 9a is the sole compound amongst the designed ones which turned out quite active against both the tested Picornaviruses (EC<sub>50</sub> values of 23 and 43 µM, respectively). Surprisingly, the mono-substituted derivatives obtained as secondary products were found more active than the corresponding dicarboxamides. Compound 4c was the most active against CV-B5, along with 4a and 4d with EC<sub>50</sub> values ranging from 9 to 13 µM. The latter two are also
endowed with poor or no cytotoxicity against Vero-76 cell line (CC$_{50}$ 90 µM and >100 µM respectively).

**Antitumor activity**

All synthesized compounds was also tested for antitumor activity against 7 cancer cell lines. For this purpose, human leukemia-lymphoma-derived cell lines CCRF-CEM, WIL-2NS, CCRF-SB and solid tumor-derived cell lines SK-MEL28, SK-MES 1, DU145, HeLa were selected. Fibroblast CRL 7065 cell line was used as control. Table 2 shows the results of the most promising compounds, derivatives 3b, 3d among bis-benzotriazole-dicarboxamides and 4d, 9b among mono-substituted acidic derivatives. In general terms, compound 3b showed the widest range of activity reducing cell proliferation of six out of seven cell lines with CC$_{50}$ ranging in the low micromolar values. Derivative 3d had the lowest CC$_{50}$ value against CCRF-CEM cell line (70 nM), while resulted inactive against most of the other tested cell lines. Among the mono-substituted acidic derivatives, 4d was proved active when tested on human leukemia-lymphoma-derived cell lines while completely inactive against solid tumors proving an interesting selectivity of action. Compound 9b is the one that turned out active against all the tested cancer cell lines, proving a wide range of action, showing, however, very low CC$_{50}$ values. Dicarboxamides turned out slightly more cytotoxic when tested on CRL 7065 control cell line than the tested mono-substituted derivatives. To determine the cytotoxic selectivity of tested compounds, the selectivity index (SI) was calculated as a ratio of CC$_{50}$ of non-tumor cells and CC$_{50}$ of tumor cells. Since the non-specific mechanism of action of designed compounds SI values turned out very low, except for compound 3d. The latter possessed CC$_{50}$ values of 70 nM against tumor CCRF-CEM cell line and 370 nM against non-tumor CRL 7065 cell line, with a resulting SI of 5.3.

The above-mentioned four promising compounds (3b, 3d, 4d, and 9b) were then subjected to the NCI60 Human Tumor Cell Lines Screen (full tables of results in Supplementary Material). This screening was performed by the National Cancer Institute (NCI, Bethesda, United States), and evaluated our compounds in the anticancer assay at 10 µM concentration against a panel of 60 human tumor cell lines. The panel comprises a series of different cancer lines comprising hematological (leukemia) tumors and solid ones (non-small cell lung - NSCL, colon, central nervous system - CNS, renal, ovarian, breast and prostate cancers and melanoma). Among the tested
compounds, 3b turned out as the most active with the widest range of action. Results from the assays are reported as Percentage of Growth Inhibition (PGI) and are graphed in bar charts depicted in Figure 4. Cell lines are grouped per type of cancer. Our lead compound 3b showed PGI values higher than 50% for 29 of the 60 cell lines, proving a wide antiproliferative activity. Best scores were recorded for breast cancer since most of the PGI values ranged from 48 up to 81%, and for prostate cancer (80 and 66% values of PGI against the two cell lines). Compound 3b can be considered as an interesting antiproliferative agent against ovarian (OVCAR-8, SK-OV-3), CNS (SF-295, SNB19, SNB75, U251) and NSCL (A549/ATCC, HOP-62, NCI-H226, NCI-H23, NCI-H460, NCI-H522) cancers, while the growth of leukemia, melanoma, renal and colon cancer cells was less affected by the administration of derivative 3b. The latter was also proved cytotoxic more than antiproliferative, against one ovarian cancer cell line (OVCAR-4) showing a PGI of 105.56%.

Apoptosis Assay
Aiming to investigate the mechanism wherewith the cells die after lead compound administration, an apoptosis assay was performed. Data plots were produced by employing normalized fluorescent expression of Annexin V and PI (Figures 5A–C). Based on intensities distribution the percentage of living, early apoptotic, late apoptotic, and necrotic cells in untreated and treated conditions were analyzed. In the untreated cells (control) the majority (91.4%) of the cells were viable, the remaining cells died in the apoptotic and necrotic way (4.4 and 4.2%, respectively) (Figure 5A). Compound 3b induced apoptosis after 96 h in a dose-dependent manner with 40% of apoptotic cells in 20 µM treated and 32% in 7 µM treated cells (Figures 5B,C, respectively). Particularly, Figures 5D,E clearly showed that most dead cells are characterized by early apoptotic features.

CONCLUSION
We have designed and synthesized 56 new compounds, 31 bis-benzotriazole dicarboxamides (3a-g, 5e-g, 7a-g, 8a-g, 9a-g) and 25 corresponding mono-substituted acidic compounds (4a-d,f, 6f, 10a-g, 11a-g, 12a-d,f) as potential antiviral and/or antitumor agents, acting as false substrates. They were purified, characterized and tested for antiviral and antitumor activity in properly selected assays. Only a few of the synthesized compounds turned out as active against Coxsackievirus B5. Compounds 4a, 4c, and 4d showed EC50 values ranging from 9 to 13 µM against CV-B5, while derivative 9a is the sole compound among the bis-benzotriazole dicarboxamides that turned out quite active against both the tested Picornaviruses with EC50 values of 23 and 43 µM, against CV-B5 and Sb-B1, respectively. From a selection of representatives that were subjected to an antitumor in vitro assay, four of them (3b, 3d, 4d and 9b) resulted as the most interesting for their CC50 values that
mostly turned out to be in the micromolar range. The same derivatives were tested by the National Cancer Institute on a panel of 60 human tumor cell lines. All the newly synthesized compounds that showed antiviral or anticancer activity bear two chlorine atoms on the BT scaffold (series 3, 4 and 9). Concerning the linker, the medium-length (−CH₂−) and the unsaturated (−CH=−) ones showed the best antitumor results (3b, 3d, 4d, and 9b), while the aromatic linkers entail a complete loss of activity. 1H-BT moity turned out to be the scaffold endowed with both biological activities (series 3, 4, and 9).

For the wide range of activity and the potency proved by the two screening assays, compound 3b was selected as lead compound. When derivative 3b was evaluated in apoptosis assay, results showed that the compound induced cell death by apoptosis in human lung tumor SK-MES 1. These findings will grant further studies involving this promising antiproliferative candidate. The latter will be used as a starting point for the next generation of compounds that will be the result of a proper structure modification process in order to increase the antiproliferative activity and potency. Alongside, compound 4c will be used as a base to design new mono-substituted acidic compounds endowed with selective anti-Coxsackievirus activity.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

**AUTHOR CONTRIBUTIONS**

SP and AC conceived and designed the experiments. RI, SP, PC, and FR synthesized the compounds. RL, ID, GC, GS, PC and TC performed antiviral, antiproliferative and apoptosis experiments. All authors analyzed the data. SP and RI wrote the paper. All authors reviewed and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2021.660424/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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