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Highlights

- 1,3,4-Oxadiazole-5-fluorocytosine hybrids were synthesized and evaluated for their anticancer activity against 4 cancer cell lines.
- Compound 5e exhibited the best antiproliferative activities against HT-1080 cells.
- Compound 5e induced apoptosis by activation of caspase 3/7 and arrested cell cycle.
- Molecular docking suggested that compound 5e activates caspase-3.
- Hybrid compounds showed no detectable anti-SARS-CoV-2 activities.
Synthesis, characterization, molecular docking, and anticancer activities of new 1,3,4-oxadiazole-5-fluorocytosine hybrid derivatives

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Abstract

Analogs of pyrimidine and 1,3,4-oxadiazole are two well established class of molecules proven as potent antiviral and anticancer agents in the pharmaceutical industry. We envisioned designing new molecules where these two heterocycles were conjugated with the goal of enhancing biological activity. In this vein, we synthesized a series of novel pyrimidine-1,3,4-oxadiazole conjugated hybrid molecules as potential anticancer and antiviral agents. Herein, we present a new design for 5-fluorocytosine-1,3,4-oxadiazole hybrids (5a-h) connected via a methylene bridge. An efficient synthesis of new derivatives was established, and all compounds were fully characterized by NMR and MS. Eight compounds were evaluated for their cytotoxic activity against fibrosarcoma (HT-1080), breast (MCF-7 and MDA-MB-231), lung carcinoma (A-549), and for their antiviral activity against SARS-CoV-2. Among all compounds tested, the compound 5e showed marked growth inhibition against all cell lines tested, particularly in HT-1080, with IC₅₀ values of 19.56 µM. Meanwhile, all tested compounds showed no anti-SARS-CoV-2 activity, with EC₅₀ >100 µM. The mechanism of cell death was investigated using Annexin V staining, caspase-3/7 activity, and analysis of cell cycle progression. The compound 5e induced apoptosis by the activation of caspase-3/7 and cell-cycle arrest in HT-1080 and A-549 cells at the G2M phase. The molecular docking suggested that the compound 5e activated caspase-3 via the formation of a stable complex protein-ligand.
Key words: 5-fluorocytosine, 1,3,4-oxadiazole hybrid, cytotoxic activity, apoptosis induction, anti-SARS-CoV-2, molecular docking.

Introduction

Search for the treatment of cancer and viral disease remains one of modern medicine's most difficult challenges. Cancer is the second leading cause of death after heart disease, accounting for one in every eight deaths worldwide [1]. Because of undesirable side effects, drug resistance, and reduced bioavailability of currently available therapeutics, the discovery of new anticancer and antiviral compounds remains a critical component in chemotherapy [2]. Apoptosis (programmed cell death) is a fundamental biological process that allows tumor cells and pathogen-infected cells to be killed and removed efficiently [3]. The sequential activation of a family of cysteinyl-aspartate-specific proteases (Caspases) mediates the apoptosis process [4]. Caspases-3 and -7 are critical effector enzymes in the execution of apoptosis [5]. As a result, apoptosis has emerged as an appealing target for the development of new anticancer agents [6].

Pyrimidine derivatives have a rich history in the field of medicinal chemistry, especially anticancer and antiviral treatment [7-12]. Moreover, being a building-block of DNA and RNA, pyrimidine derivatives were found to be associated with a variety of chemotherapeutic effects including antimicrobial [13], anti-tubercular [14], antifungal [15], and antimalarial [16] activities. Capecitabine I is an anticancer drug containing 5-fluorocytosine pyrimidine base which is an efficient inhibitor of colon cancer cell line HCT-15 through the mitochondrial pathway of apoptosis [17]. Additionally, capecitabine is known to induce apoptosis through the activation of caspase-3 in colorectal cancer caco-2 cell lines [18]. Furthermore, dideoxy-2′-fluoro-4′-thionucleosides 5-fluorocytosine II exhibited potent anti-HIV activity with an EC50 of 0.15 μM [19]. The carbocyclic nucleoside of 5-fluorocytosine and cytosine III showed high selectivity against Venezuela equine encephalitis virus (VEEV) and various strains of H5N1 influenza [20].

1,3,4-Oxadiazole is another useful scaffold in medicinal chemistry offering both versatility and structural diversity for the design of drug molecules. In some cases, it serves as a bioisostere for carbonyl-containing compounds like esters, amides, and carbamates, or as a flat aromatic linker to provide the molecular geometry. Stability of the oxadiazole ring and its ability to interact with bio-targets via $\pi-\pi$ interaction and strong hydrogen bonding justifies our interest in developing bioactive molecules containing this scaffold [21-24]. Stecoza and
coworkers have synthesized a series of new 2,5-diaryl/heteroaryl-1,3,4-oxadiazoles IV, which showed promising cytotoxicity against HT-29 (colon adenocarcinoma) and MDA-MB-231 (breast adenocarcinoma). 1,3,4-Oxadiazole compounds IV were able to arrest the cell cycle and induced apoptosis in the MDA-MB-231 cell lines [25]. Additionally, a series of new derivatives bearing 1,3,4-oxadiazole moiety V were designed, synthesized, and evaluated for their anticancer activities in four human cancer cell lines (HepG2, A549, MCF-7, and HCT-116). These compounds exhibited significant cytotoxic activity with mitochondrial-mediated apoptosis by decreasing mitochondrial membrane potential, up-regulation of Bax, down-regulation of Bcl-2 and activation levels of the caspase cascade [26]. Cancer patients are particularly vulnerable to coronavirus (COVID-19) [27, 28]. These individuals are not only more susceptible to this infection, but also more frequently develop severe pneumonia during the course of disease [29]. However, limited information is known about the outcome of chemotherapy for cancer patients with prior COVID-19 infection [30]. Meanwhile, Rabie et al. reported that the oxadiazole VI and VII exhibited significant anti-COVID-19 activity (anti-SARS-CoV-2 EC50 = 0.31 and 1.01 μM, respectively), which could be promising lead compound for the design of new anti-COVID-19 agents [31]. They also proposed two new 1,3,4-oxadiazole compounds CoViTris2020 and ChloViD2020 as the first multitarget coronaviral protein blockers with high potency [32].

Recently, we have synthesized several novel pyrimidine-1,3,4-oxadiazole hybrids with potential anticancer and antiviral agents [33-36]. Continuing our effort to discover bioactive compounds, herein, we report the design and synthesis of new 5-fluorocytosine-1,3,4-oxadiazole hybrids (5a-h) conjugated through a methylene bridge. The prepared products were evaluated for their anticancer activities in HT-1080, A-549, MCF-7, and MDA-MB-231 cell lines. We studied the possible mechanism of apoptosis induction mechanism and the activation of caspase-3/7. Molecular docking study was carried out to investigate the ability of compound 5e to activate the caspase-3 protein. Finally, all compounds (5a-h) were tested against anti-COVID-19 activity against Beta Cov / Belgium / GHB-03021/2020.
**Figure 1: Design of the new 1,3,4-oxadiazole-5-fluorocytosine hybrid derivatives**

**Materials & Methods**

**Chemistry**

**General**

Melting points were measured using a Büchi B-545 digital capillary melting point apparatus and used without correction. Reactions were checked with TLC using aluminum sheets with silica gel 60 F254 from Merck. IR Spectra were recorded on a Perkin-Elmer VERTEX 70 FTIR spectrometer covering field 400-4000 cm\(^{-1}\). The \(^1\)H NMR and \(^{13}\)C NMR spectra were recorded in DMSO-d\(_6\) or CDCl\(_3\) on a Bruker Advance 300 spectrometer at 300 and 75 MHz, respectively. The chemical shifts are expressed in parts per million (ppm) by using DMSO-d\(_6\) as internal reference. The multiplicities of the signals are indicated by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quadruplet; and m, multiplet.
coupling constants $J$ are expressed in Hertz. Mass spectra were collected using an API 3200 LC/MS/MS system, equipped with an ESI source. The chemical reagents used in synthesis were purchased from Fluka, Sigma and Aldrich.

**Preparation of alkylating agent 4a-j**

First, 10 mmol of individual carboxylic acid 1a-g was taken in 15 ml of methanol, and thionyl chloride was added dropwise over 10 min at 0 °C. Then, the mixture was stirred at room temperature for 3 hours, methanol was evaporated under vacuum, extracted with ethyl acetate, and purified by column chromatography to afford esters 2a-g. In the second step, to a round bottom flask (25 mL) equipped with a condenser, individual ester 2a-g (10 mmol), hydrazine hydrate (11 mmol, 0.5 ml) and ethanol (5 ml) were added. The mixture was refluxed for 3 hours. The solvent was evaporated under vacuum and the hydrazide was recrystallized using ethanol to afford the desired product 3a-g. Then, chloroacetic acid (11 mmol, 1.04 g), phosphorus oxychloride (30 mmol, 2.8 ml) and DCE (20 ml) were mixed in 100 ml round bottom flask and heated at 80 °C for 2 hours. Next, each carboxylic acid hydrazide (2a-g) was added to the mixture and heated for 12 hours. After completion of the reaction, water (40 ml) was added, and the pH was adjusted to 7–8 with sodium hydrogen carbonate and extracted with dichloromethane. The organic layer was dried (Na$_2$SO$_4$), evaporated, and purified by column chromatography (silica gel, 5% EtOAc in hexane) to give alkylating agents 4a-g.

The alkylating agents 4h-j were prepared by similar procedure. The only difference is the products 1h-i (10 mmol) were subjected to the benzylation reaction. This reaction was carried out using 11 mmol of benzyl and 4-fluorobenzyl chloride, 10 mmol of potassium carbonate, and 20 ml of acetone at reflux for 12 hours. Compounds 4h-j were purified by column chromatography using mixture of hexane and ethyl acetate.

**2-(Chloromethyl)-5-phenyl-1,3,4-oxadiazole (4a)**

Rf = 0.4 (9/1 (Hexane/AcOEt)). IR U (KBr, cm$^{-1}$): 3029 (Csp2), 2972 (Csp3), 1552 (C=N), 1017 (C-O-C), 746 (C-Cl). $^1$H NMR (300 MHz, CDCl$_3$) δ (ppm): 5.16 (s, 2H, CH$_2$); 7.6 (m, 3H, H$_{Ph}$); 8.03 (m, 2H, H$_{Ph}$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ (ppm): 33.23 (CH$_2$); 122.85 (C$_{Ph,IV}$); 126.65 (C$_{Ph,III}$); 129.53 (C$_{Ph,III}$); 132.38 (C$_{Ph,II}$); 162.88, 164.95 (Coxa, Coxa).

**2-(Chloromethyl)-5-(2-chlorophenyl)-1,3,4-oxadiazole (4b)**

Rf = 0.35 (9/1 (Hexane/AcOEt)). IR U (KBr, cm$^{-1}$): 3018 (Csp2), 2964 (Csp3), 1554 (C=N), 1013 (C-O-C), 753 (C-Cl). $^1$H NMR (300 MHz, CDCl$_3$) δ (ppm): 5.22 (s, 2H, CH$_2$); 7.5 (d,
\[ \delta_{1H} (ppm): 2.49 (s, 3H, CH3); 5.58 (s, 1H, HPh); 7.29 (d.d, 3J_H-H = 7.5 Hz et 3J_H-H = 7.2 Hz, 1H, HPh); 7.93 (d, 3J_H-H = 7.5 Hz, 1H, HPh). \]
\[ ^{13}C \text{ NMR}(75 \text{ MHz, CDCl}_3) \delta (ppm): 35.41 (CH2); 122.65 (C(Ph,IV)); 127.82 (C(Ph,III)); 131.52 (C(Ph,III)); 131.83 (C(Ph,III)); 132.37 (C(Ph,III)); 133.98 (C(Ph,IV)); 162.35, 164.22 (Coxa, Coxa). \]

2-(Chloromethyl)-5-(3-chlorophenyl)-1,3,4-oxadiazole (4c)

Rf = 0.37 (9/1 (Hexane/AcOEt)). IR (KBr, cm\(^{-1}\)): 3015 (Csp2), 2968 (Csp3), 1562 (C=N), 1021 (C-O-C), 758 (C-Cl). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta (ppm): 5.19 \) (s, 2H, CH\(_2\)); 7.63 (m, 2H, HPh); 7.92 (m, 2H, HPh). \(^{13}C \text{ NMR}(75 \text{ MHz, CDCl}_3) \delta (ppm): 37.52 \) (CH2); 124.84 (C(Ph,IV)); 125.14 (C(Ph,III)); 125.89 (C(Ph,III)); 131.62 (C(Ph,III)); 132.02 (C(Ph,III)); 133.17 (C(Ph,IV)); 162.53, 164.47 (Coxa, Coxa).

2-(Chloromethyl)-5-(3-nitrophenyl)-1,3,4-oxadiazole (4d)

Rf = 0.22 (9/1 (Hexane/AcOEt)). IR (KBr, cm\(^{-1}\)): 3012 (Csp2), 2972 (Csp3), 1589 (NO\(_2\)), 1574 (C=N), 1028 (C-O-C), 755 (C-Cl). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta (ppm): 5.19 \) (s, 2H, CH\(_2\)); 7.63 (t, 3J\(_{H-H} = 7.8 \) Hz, 1H, HPh); 8.45 (m, 2H, HPh); 8.92 (t, 4J\(_{H-H} = 1.8 \) Hz, 1H, HPh). \(^{13}C \text{ NMR}(75 \text{ MHz, CDCl}_3) \delta (ppm): 32.79 \) (CH2); 122.06 (C(Ph,III)); 125.00 (C(Ph,IV)); 126.61 (C(Ph,III)); 130.56 (C(Ph,III)); 132.60 (C(Ph,III)); 162.95, 164.12 (Coxa, Coxa).

2-(Chloromethyl)-5-(4-chlorophenyl)-1,3,4-oxadiazole (4e)

Rf = 0.34 (9/1 (Hexane/AcOEt)). IR (KBr, cm\(^{-1}\)): 3021 (Csp2), 2964 (Csp3), 1568 (C=N), 1011 (C-O-C), 756 (C-Cl). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta (ppm): 5.15 \) (s, 2H, CH\(_2\)); 7.66 (t, 3J\(_{H-H} = 7.5 \) Hz, 2H, HPh); 8.01 (d, 3J\(_{H-H} = 7.5 \) Hz, 2H, HPh). \(^{13}C \text{ NMR}(75 \text{ MHz, CDCl}_3) \delta (ppm): 36.23 \) (CH2); 121.09 (C(Ph,IV)); 124.62 (C(Ph,III)); 125.97 (C(Ph,III)); 136.45 (C(Ph,IV)); 162.28, 164.12 (C-2, C-3).

2-(Chloromethyl)-5-(4-fluorophenyl)-1,3,4-oxadiazole (4f)

Rf = 0.31 (9/1 (Hexane/AcOEt)). IR (KBr, cm\(^{-1}\)): 3014 (Csp2), 2961 (Csp3), 1584 (C=N), 1079 (C-O-C), 1003 (C-F), 760 (C-Cl). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta (ppm): 4.79 \) (s, 2H, CH\(_2\)); 7.25 (m, 2H, HPh); 8.12 (m, 2H, HPh). \(^{13}C \text{ NMR}(75 \text{ MHz, CDCl}_3) \delta (ppm): 32.96 \) (CH2); 116.50 (d, 2J\(_{C-F} = 22 \) Hz, C(Ph,III)); 119.68 (d, 4J\(_{C-F} = 3 \) Hz, C(Ph,IV)); 129.47 (d, 3J\(_{C-F} = 9 \) Hz, C(Ph,III)); 163.72 (d, 1J\(_{C-F} = 228 \) Hz, C(Ph,IV)); 163.40, 166.77 (Coxa, Coxa).

2-(Chloromethyl)-5-(4-methylphenyl)-1,3,4-oxadiazole (4g)

Rf = 0.46 (9/1 (Hexane/AcOEt)). IR (KBr, cm\(^{-1}\)): 3020 (Csp2), 2965 (Csp3), 1562 (C=N), 1018 (C-O-C), 760 (C-Cl). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta (ppm): 2.49 \) (s, 3H, CH\(_2\)); 5.58 (s, 2H, HPh).
2H, H-1); 7.39 (d, $^3J_{H-H} = 8.1$ Hz, 2H, HPh); 7.82 (d, $^3J_{H-H} = 8.1$ Hz, 2H, HPh). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ (ppm): 21.64 (CH$_3$); 39.12 (CH$_2$); 115.04 (C$_{Ph,IV}$); 126.87 (C$_{Ph,III}$); 130.50 (C$_{Ph,III}$); 132.85 (C$_{Ph,IV}$); 162.81, 164.77 (Coxa, Coxa).

2-(Chloromethyl)-5-(4-(4-fluorobenzyl)oxyphenyl)-1,3,4-oxadiazole (4h)

Rf = 0.39 (9/1 (Hexane/AcOEt)). IR U (KBr, cm$^{-1}$): 3078 (Csp2), 2986 (Csp3), 1563 (C=N), 1018 (C-O-C), 760 (C-Cl). $^1$H NMR (300 MHz, DMSO-d6) $\delta$ (ppm): 5.19 (s, 2H, CH$_2$); 5.61 (s, 2H, CH$_2$); 7.18 (d, $^3J = 8.7$ Hz, 4H, HPh); 7.55 (d, $^3J = 6.6$ Hz, 2H, 2HPh); 7.93 (d, 2H, 2HPh). $^{13}$C NMR (75 MHz, DMSO-d6) $\delta$(ppm): 22.08 (CH$_2$); 69.02 (CH$_2$); 115.42 (d, $^2J_{C-F} = 21$ Hz, C$_{Ph,III}$); 115.69 (C$_{Ph,III}$); 115.81 (C$_{Ph,III}$); 128.30 (C$_{Ph,IV}$); 132.56 (d, $^4J_{C-F} = 3$ Hz, C$_{Ph,III}$); 159.85 (C$_{Ph,IV}$-O); 162.51 (d, $^1J_{C-F} = 225$ Hz, C$_{Ph,IV}$-F); 162.78, 164.52 (Coxa, Coxa).

Synthesis of homonucleosides (5a-h)

A mixture of 5-fluorocytosine (1 mmol), alkylating agent (0.75 mmol) and potassium carbonate (0.5 mmol) in DMF (10 ml) were heated at 60°C for 2 hours. The solvent was evaporated, and residue was purified by column chromatography with CH$_2$Cl$_2$ / MeOH (95/5) to obtain pure products (5a-h).

4-Amino-5-fluoro-1-((5-phenyl-1,3,4-oxadiazol-2-yl)methyl)pyrimidin-2(1H)-one (5a)

Rf = 0.43 (9/1 (CH$_2$Cl$_2$/MeOH)). Mp (°C) 267–269. IR U (KBr, cm$^{-1}$): 3348 (NH); 3070 (Csp2); 1687 (C=O); 1640 (C=O); 1138 (C-F). $^1$H NMR (300 MHz, DMSO-d6) $\delta$ (ppm): 5.17 (s, 2H, CH$_2$); 7.57 (m, 4H, NH + 3HPh); 7.90 (s, 1H, NH); 7.98 (m, 2H, 2HPh); 8.15 (d, $^3J_{H-F} = 6.6$ Hz, 1H, H-6). $^{13}$C NMR (75 MHz, DMSO-d6) $\delta$(ppm): 43.39 (CH$_2$); 123.05 (C$_{Ph,IV}$); 126.47 (C$_{Ph,III}$); 126.47 (C$_{Ph,IV}$); 129.48 (C$_{Ph,III}$); 130.36 (d, $^2J_{C-F} = 39$ Hz, C-6); 135.72 (d, $^1J_{C-F} = 240$ Hz, C-5); 153.74 (C-2); 158.07 (d, $^2J_{C-F} = 13$ Hz, C-4); 163.10, 164.14 (Coxa, Coxa). HRMS for C$_{13}$H$_{14}$FN$_2$O$_2$ calculated 288.0891, found 288.0891.

4-Amino-1-((5-(2-chlorophenyl)-1,3,4-oxadiazol-2-yl)methyl)-5-fluoropyrimidin-2(1H)-one (5b)

Rf = 0.45 (9/1 (CH$_2$Cl$_2$/MeOH)). Mp (°C) 242–244. IR U (KBr, cm$^{-1}$): 3348 (NH); 3069 (Csp2); 1687 (C=O); 1642 (C=O); 1139 (C-F); 743 (C-Cl). $^1$H NMR (300 MHz, DMSO-d6) $\delta$(ppm): 5.20 (s, 2H, CH$_2$); 7.57 (t, $^3J_{H-H} = 7.2$ Hz, 1H, HPh); 7.62 (m, 3H, 2HPh + NH); 7.90 (s, 1H, NH); 7.94 (d.d, $^3J_{H-H} = 7.8$ Hz et $^3J_{H-H} = 7.5$ Hz, 1H, HPh); 8.14 (d, $^3J_{H-F} = 6.6$ Hz, 1H, H-6). $^{13}$C NMR (75 MHz, DMSO-d6) $\delta$(ppm): 43.39 (CH$_2$); 122.24 (C$_{Ph,IV}$); 127.1 (C$_{Ph,III}$); 130.12 (C$_{Ph,IV}$); 130.54 (C$_{Ph,III}$); 131.13 (C$_{Ph,III}$); 131.46 (d, $^2J_{C-F} = 39$ Hz, C-6); 133.32
(CPh,III); 135.84 (d, $^1J_{CF} = 240$ Hz, C-5); 153.75 (C-2); 158.08 (d, $^2J_{CF} = 13$ Hz, C-4); 162.28, 163.48 (Coxa, Coxa). HRMS for $\text{C}_{13}\text{H}_{10}\text{ClF_N}_2\text{O}_2^+$ calculated 322.0502, found 322.0506.

4-Amino-1-((5-(3-chlorophenyl)-1,3,4-oxadiazol-2-yl)methyl)-5-fluoropyrimidin-2(1H)-one (5c)

$R_f = 0.44$ (9/1 (CH$_2$Cl$_2$/MeOH)). Mp (°C) 264–266. $^1$H NMR (300 MHz, DMSO-d$_6$) δ (ppm): 5.18 (s, 2H, CH$_2$); 7.66 (m, 3H, 2H$_{Ph} +$ NH); 7.89 (s, 1H, NH); 7.94 (m, 2H, H$_{Ph}$); 8.14 (d, $^3J_{H\cdotp H} = 6.9$ Hz, 1H, H-6). $^1$H NMR (300 MHz, DMSO-d$_6$) δ (ppm): 33.07 (CH$_2$); 124.97 (C$_{Ph,IV}$); 125.20 (C$_{Ph,III}$); 125.94 (C$_{Ph,II}$); 130.83 (d, $^2J_{CF} = 31$ Hz, C-6); 131.53 (C$_{Ph,III}$); 131.92 (C$_{Ph,III}$); 134.06 (C$_{Ph,IV}$); 135.85 (d, $^1J_{CF} = 240$ Hz, C-5); 153.73 (C-2); 158.08 (d, $^2J_{CF} = 13$ Hz, C-4); 163.05, 163.46 (Coxa, Coxa). IR U (KBr, cm$^{-1}$): 3068 (Csp2); 1600 (C=O); 1640 (C=O); 1140 (C-F); 743 (C-Cl). HRMS for $\text{C}_{13}\text{H}_{10}\text{ClF_N}_2\text{O}_2^+$ calculated 322.0502, found 322.0510.

4-Amino-1-((5-(3-nitrophenyl)-1,3,4-oxadiazol-2-yl)methyl)-5-fluoropyrimidin-2(1H)-one (5d)

$R_f = 0.41$ (9/1 (CH$_2$Cl$_2$/MeOH)). Mp (°C) 251–253. $^1$H NMR (300 MHz, DMSO-d$_6$) δ (ppm): 5.22 (s, 2H, CH$_2$); 7.66 (s, 1H, NH); 7.90 (m, 2H, H$_{Ph} +$ NH); 8.16 (d, $^3J_{H\cdotp H} = 6.6$ Hz, 1H, H-6); 8.40 (d, $^3J_{H\cdotp H} = 7.8$ Hz et $^4J_{H\cdotp H} = 1.2$ Hz, 1H, H$_{Ph}$); 8.47 (m, 1H, H$_{Ph}$). $^1$C NMR (300 MHz, DMSO-d$_6$) δ (ppm): 43.35 (CH$_2$); 120.99 (C$_{Ph,IV}$); 124.49 (C$_{Ph,IV}$); 126.45 (C$_{Ph,III}$); 130.83 (d, $^2J_{CF} = 32$ Hz, C-6); 131.42 (C$_{Ph,III}$); 132.55 (C$_{Ph,III}$); 135.86 (d, $^1J_{CF} = 240$ Hz, C-5); 148.21 (C$_{Ph,IV}$); 153.74 (C-2); 158.09 (d, $^2J_{CF} = 13$ Hz, C-4); 162.7, 163.79 (Coxa, Coxa). IR U (KBr, cm$^{-1}$): 3347 (NH); 3069 (Csp2); 1686 (C=O); 1641 (C=O); 1573 (N-O); 1139 (C-F). HRMS for $\text{C}_{13}\text{H}_{10}\text{F_N}_6\text{O}_4^+$ calculated 333.0742, found 333.0732.

4-Amino-1-((5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)methyl)-5-fluoropyrimidin-2(1H)-one (5e)

$R_f = 0.43$ (9/1 (CH$_2$Cl$_2$/MeOH)). Mp (°C) 317–319. $^1$H NMR (300 MHz, DMSO-d$_6$) δ (ppm): 5.17 (s, 2H, CH$_2$); 7.67 (m, 3H, 2H$_{Ph} +$ NH); 7.89 (s, 1H, NH); 7.97 (d, $^3J_{H\cdotp H} = 8.4$ Hz, 2H, H$_{Ph}$); 8.13 (d, $^3J_{H\cdotp H} = 6.9$ Hz, 1H, H-6). $^1$C NMR (300 MHz, DMSO-d$_6$) δ (ppm): 43.37 (CH$_2$); 121.93 (C$_{Ph,IV}$); 128.29 (C$_{Ph,III}$); 129.65 (C$_{Ph,III}$); 130.33 (d, $^2J_{CF} = 31$ Hz, C-6); 135.83 (d, $^1J_{CF} = 240$ Hz, C-5); 136.80 (C$_{Ph,IV}$); 153.73 (C-2); 158.08 (d, $^2J_{CF} = 13$ Hz, C-4); 163.27, 163.41 (Coxa, Coxa). IR U (KBr, cm$^{-1}$): 3348 (NH); 3069 (Csp2); 1686(C=O); 1640(C=O); 1140 (C-F); 744 (C-Cl). HRMS for $\text{C}_{13}\text{H}_{10}\text{ClF_N}_2\text{O}_2^+$ calculated 322.0502, found 322.0502.

4-Amino-1-((5-(4-toluyl)-1,3,4-oxadiazol-2-yl)methyl)-5-fluoropyrimidin-2(1H)-one (5f)
Rf = 0.17 (9.5/0.5 (CH₂Cl₂/MeOH)). Mp (°C) 271–273. ⋅H NMR (300 MHz, DMSO-d₆) δ (ppm): 2.39 (CH₃); 5.16 (s, 2H, CH₂); 7.41 (d, 2H, 2Hₚₜ); 7.65 (s, 1H, NH); 7.97 (m, 3H, NH + 2Hₚₜ); 8.14 (d, 3 JₜₓF = 6.9 Hz, 1H, H-6). ¹³C NMR (75 MHz, DMSO-d₆) δ (ppm): 43.39(CH₂); 123.05 (Cₚₚ,IV); 126.47 (Cₚₚ,III); 129.48 (Cₚₚ,III); 130.64 (d, 2 JₖF = 31 Hz, C-6); 132.08 (Cₚₚ,IV); 135.85 (d, 1 JₗC-F = 240 Hz, C-5); 135.74 (C-2); 158.13 (d, 2 JₖF = 13 Hz, C-4); 163.10, 163.14 (C₀xa, C₀xa).IR U (KBr, cm⁻¹): 3348 (NH); 3067 (Cₛp²); 1686 (C=O);1639 (C=O); 1184 (C-F); HRMS for C₁₄H₁₃F₅N₅O₂⁺ calculated 302.1048, found 302.1044.

4-Amino-1-((5-(4-fluorophenyl)-1,3,4-oxadiazol-2-yl)methyl)-5-fluoropyrimidin-2(1H)-one (5g)

Rf = 0.40 (9/1 (CH₂Cl₂/MeOH)). Mp (°C) 293–295. ⋅H NMR (300 MHz, DMSO-d₆) δ (ppm):5.17 (s, 2H, CH₂); 7.45 (t, 3 J = 7.2 Hz, 2H, 2Hₚₜ); 7.65 (s, 1H, NH); 7.88 (s, 1H, NH); 8.03 (m, 2H, Hₚₜ); 8.13 (d, 3 JₜₓF = 6.6 Hz,1H, H-6). ¹³C NMR (75 MHz, DMSO-d₆) δ (ppm):43.35 (CH₂);116.73 (d, 2 JₗC-F = 22 Hz, Cₚₚ,III); 119.74 (d, 4 JₕC-F = 3 Hz, Cₚₚ,IV); 129.22 (d, 3 JₗC-F = 9.15 Hz, Cₚₚ,III);130.33 (d, 2 JₕC-F = 34 Hz, C-6); 135.83 (d, 1 JₕC-F = 240 Hz, C-5); 153.73 (C-2); 158.08 (d, 2 JₗC-F = 13 Hz, C-4):163.11, 163.42 (C₀xa, C₀xa); 164.11 (d, 1 JₕC-F = 249 Hz, Cₚₚ,IV).IR U (KBr, cm⁻¹): 3349 (NH); 3067 (Cₛp²); 1684(C=O);1639 (C=O); 1185 (C-F); HRMS for C₁₃H₁₀F₂N₅O⁺ calculated 306.0797, found 306.0799.

4-Amino-5-fluoro-1-((5-(4-fluorobenzyl)oxy)phenyl)-1,3,4-oxadiazol-2-(1H)-one (5h)

Rf = 0.46 (9/1 (CH₂Cl₂/MeOH)). Mp (°C) 290-292. ⋅H NMR (300 MHz, DMSO-d₆) δ (ppm): 5.15 (s, 2H, CH₂); 5.18 (s, 2H, CH₂); 7.23 (d, 3 J = 9 Hz, 4H, Hₚₜ); 7.54 (m, 2H, 2Hₚₜ); 7.65 (s, 1H, NH); 7.90 (m, 3H, 2Hₚₜ + NH); 8.14 (d, 3 JₜₓF = 6.6 Hz,1H, H-6). ¹³C NMR (75 MHz, DMSO-d₆) δ (ppm): 43.35 (CH₂); 68.78 (CH₂); 115.29 (d, 2 JₕC-F = 21 Hz, Cₚₚ,III); 115.64 (Cₚₚ,III); 115.67 (Cₚₚ,III); 128.23 (Cₚₚ,IV); 130.34 (d, 2 JₕC-F = 33 Hz, C-6); 132.62 (d, 4 JₕC-F = 3 Hz, Cₚₚ,III); 135.83 (d, 1 JₕC-F = 240 Hz, C-5); 153.74 (C-2); 158.08 (d, 2 JₕC-F = 13 Hz, C-4); 160.23 (Cₚₚ,IV-O); 162.53 (d, 1 JₕC-F = 225 Hz, Cₚₚ,IV-F); 162.52, 163.46 (C₀xa, C₀xa).IR U (KBr, cm⁻¹): 3349 (NH); 3067 (Cₛp²); 1684 (C=O); 1639 (C=O); 1286 (C-O); 1185 (C-F); 1117 (C-O). HRMS for C₂₉H₁₆F₂N₅O₃⁺ calculated 412.1216, found 412.1208.

Biology

Anti-SARS-CoV-2
The SARS-CoV-2 isolate used was derived from the BetaCov/Belgium/GHB-03021/2020 (EPI ISL407976|2020-02-03), which was isolated from a Belgian patient returning from Wuhan in February 2020. The isolate was passaged 7 times on VeroE6 cell lines which introduced two series of amino acid deletions in the spike protein [37]. The infectious content of the virus stock was determined by titration on Vero E6 cell lines.

The SARS-CoV-2 antiviral assay is based on the previously established SARS-CoV assay [38]. Upon infection the fluorescence of VeroE6-GFP cell cultures declines due to the cytopathogenic effect of the replicating virus. In the presence of an antiviral compound, the cytopathogenicity is inhibited and the fluorescent signal maintained. To this end VeroE6-GFP cell lines (kindly provided by Marnix Van Loock, Janssen Pharmaceutica, Beerse, Belgium), were used as described previously [39, 40]. Since VeroE6 cell lines show a high efflux of some chemotypes, the antiviral assays were performed in the presence of the P-glycoprotein (Pgp) efflux inhibitor CP-100356 (0.5 μM) [41].

**Anticancer activity evaluation**

**Cell culture**

Four cancer cell lines: HT-1080 fibrosarcoma, A-549 lung carcinoma, MCF-7, and MDA-MB-231 breast adenocarcinoma were kindly provided by Dr. P. Coursaget from INSERM (Tours, France). A-549, MCF-7, and MDA-MB-231 cell lines were cultured in DMEM, whereas HT-1080 cell lines were cultured in MEM culture media, both supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

**Cytotoxicity and antiproliferative activity**

Cells were seeded in 96-well plates at a density of 5x10³ cells/well for 24 hours. Subsequently, cells were treated with various concentrations of the compounds 5a-h for 24 hours. Doxorubicin (TEVA Pharma S.A., Courbevoie, France) was used as a positive control. Cell growth was analyzed using MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Cells were incubated with 5 mg/mL of MTT for 4h at +37 °C. The supernatant was then discarded before dissolving the product of the reaction formazan with 150µL of DMSO. The absorbance was then measured at 570 nm wavelength using a microplate reader (Thermo Scientific, Paris, France). IC₅₀ was estimated using Graph Pad Prism7.
Annexin V binding assay

Cells were seeded at a density of 2×10^5/wells and incubated overnight at 37 °C. The cells were then treated with the compound 5e at concentration of 20 µM for 24 hours. Cells were then harvested and washed twice with phosphate-buffered saline (PBS) at 4 °C. Cells were centrifuged at 300 g for 5 min and washed twice with PBS at 4 °C before adding 100 µL of Annexin V Binding Buffer staining (Annexin V Apoptosis Detection KIT with 7-AAD, Millipore-Merck, Fontenay sous Bois, France). The cells were incubated for 20 min at room temperature in the dark. Apoptotic cells were evaluated using a Muse Cell Analyzer (Millipore-Merck).

Caspase-3/7 activity

Cells were treated with 20 µM concentration of the compound 5e. After 24 hours incubation, cells were harvested and washed twice with PBS at 4 °C. After staining with the Caspase assay kit (Promega) and incubation for 30 min, caspase-3 activity was analyzed according to the manufacturer instructions using the Muse Cell Analyzer (Millipore-Merck).

Cell cycle assay

Cells were treated with 20µM of the compound 5e for 24 hours. The cells were then harvested and washed with PBS. The collected cells (10^6 cells) were suspended into ice-cold ethanol 70% at -20 °C for fixation. After washing, the cells were pelleted by gentle centrifugation and suspended in 1 mL of staining buffer (100 mM Tris, pH = 7.4, 150 mM NaCl, 1mM CaCl₂, 0.5 mM MgCl₂, 0.1% Nonidet P-40) containing 3 µM propidium iodide (PI) (Millipore-Merck). The cells were then incubated for 15 min at room temperature and analyzed for cell cycle phase distribution using the Muse Cell Analyzer (Millipore-Merck).

Molecular docking

In silico computational docking studies were performed using AutoDock 4.2 [42, 43]. The X-ray crystallographic structure of caspase-3 was downloaded from the RCSB Protein Data Bank (PDB) ID: 6CKZ [44]. The protein was prepared separately by removing water and co-crystalized ligands bound with the protein to make receptor free of any ligand before docking. Polar hydrogen and Gastieger charges were added using the MGL tools and protein saved in PDBQT format. Ligand structure was created separately using ChemDraw Ultra 12.0, energy minimized in Chem3D, torsional bonds of ligands were set flexible and saved in PDBQT format [45]. The receptor was kept rigid and the grid covering all amino acid residues present
inside the active site of protein was built (grid box size of 50 Å x 50 Å x 50 Å with a spacing of 0.375 Å between the grid points and centered at 35.58 (x), 94.397 (y) and 17.54 (z)). The best conformers were searched by the Lamarckian genetic algorithm (LGA), the population size was set to 150 and the maximum number of energy evaluation was set to 2500000. Finally, the results were analyzed and visualized by discovery studio.

Results and discussions

Chemistry

The reaction sequences of the prepared products are summarized in Schemes 1-3. First, the esterification of benzoic acid derivatives 1a-g was carried out using thionyl chloride and methanol to obtain esters 2a-g in 87-97% yields. Then, compounds 2a-g were reacted with hydrazine in ethanol as a solvent to afford hydrazides 3a-g. Oxadiazoles 4a-g were synthesized via condensation of hydrazides 3a-g and chloroacetic acid in the presence of POCl₃ and dichloroethane at 80 °C. The 4-fluorobenzylxy (OCH₂-Ph) has been incorporated in the para position of phenyl ring to synthesize other oxadiazole derivatives (4a-g). The 4-hydroxybenzoic acid was esterified with methanol. The obtained product 1h was subjected to benzylation with 4-fluorobenzyl bromide to form the compound 2h. The latter was subjected to hydrazinolysis in ethanol to afford the hydrazide 3h. Finally, we obtained the alkylating agent 4h by reacting product 3h and chloroacetic acid in the presence of POCl₃.

Scheme 1: Synthesis of alkylating agents 4a-g

|   | R | H  | 2-Cl | 3-Cl | 3-NO₂ | 4-Cl | 4-F  | 4-CH₃ |
|---|---|----|------|------|-------|------|------|------|
| Yield (%) |   |    |      |      |       |      |      |      |
| 2 | 94 | 95 | 93   | 87   | 92    | 91   | 93   |
| 3 | 70 | 76 | 71   | 65   | 73    | 79   | 77   |
| 4 | 68 | 70 | 66   | 59   | 68    | 72   | 75   |

Scheme 1: Synthesis of alkylating agents 4a-g
We used the modified Hilbert–Johnson reaction to synthesize the new 5-fluorocytosine-1,3,4-oxadiazole hybrid conjugates (5a-h) [46]. This method afforded the desired product 5a in a low yield (12%). The reaction yield was improved when a basic medium (K₂CO₃) in dimethylformamide was employed for the conjugation step [45]. The 5-fluorocytosine-1,3,4-oxadiazole derivatives (5a-h) were obtained in 45-53% yield. Molecules 5a-h were fully characterized by ¹H NMR, ¹³C NMR, DEPT, HRMS, and FTIR.

**Scheme 3: Synthesis of 1,3,4-oxadiazole hybrids 5a-h**

**Biology**
Anti-proliferative activity

The *in-vitro* antiproliferative activities of hybrids 5a-h against four human cancer cell lines fibrosarcoma (HT-1080), breast (MCF-7 and MDA-MB-231), and lung carcinoma (A-549) were evaluated by the MTT assay using DOX as a positive control [47-49]. The IC$_{50}$ values are listed in Table 1. Most compounds showed more antiproliferative activity in HT-1080 and A-549 cell lines. The compounds 5e and 5f displayed promising antiproliferation properties for both of the above-mentioned cell lines (Table 1). Meanwhile, oxadiazole 5g containing the fluorine atom at para-position of the phenyl moiety was the most potent against MCF-7 cell lines (21.3 ± 2.18). In MDA-MB-231 lines, the benzyloxy derivative 5h showed the highest activity in the MDA-MB-231 cell lines, with an IC$_{50}$ value of 21.66 ± 0.95 µM. In summary, compound 5e was found to be the most potent with IC$_{50}$ of 19.56 ± 1.62 µM and its mechanism of action was studied using molecular docking study.

Table 1: *In-vitro* anticancer activity (IC$_{50}$) of 1,3,4-oxadiazole-5-fluorocytosine hybrids (8a-i) against HT1080, A549, MCF7 and MDA-MB231

| Product | HT-1080  | A-549   | MCF-7     | MDA-MB-231 |
|---------|----------|---------|-----------|------------|
| 5a      | 51.07 ± 5.32 | 39.41 ± 1.85 | 42.63 ± 7.11 | 63.14 ± 4.19 |
| 5b      | 67.14 ± 3.66 | 46.64 ± 3.05 | 50.32 ± 1.63 | 63.73 ± 4.08 |
| 5c      | 50.17 ± 2.42 | 67.04 ± 6.35 | 48.43 ± 6.07 | 58.22 ± 9.46 |
| 5d      | 31.44 ± 1.88 | 26.9 ± 2.34 | 21.39 ± 1.50 | 25.94 ± 2.25 |
| 5e      | 19.56 ± 1.62 | **20.08 ± 2.83** | 27.18 ± 1.95 | 23.98 ± 4.27 |
| 5f      | 20.87 ± 2.04 | 27.33 ± 5.91 | 30.43 ± 3.09 | 35.31 ± 2.66 |
| 5g      | 37.76 ± 0.25 | 25.87 ± 3.11 | **21.3 ± 2.18** | 22.74 ± 2.41 |
| 5h      | 21.88 ± 1.25 | 25.45 ± 2.04 | 30.24 ± 2.87 | **21.66 ± 0.95** |
| DOX     | 6.24 ± 0.96  | 5.30 ± 0.51   | 4.84 ± 0.31  | 4.05 ± 0.67  |

Anti-SARS-CoV-2

SARS-CoV-2 is a positive-sense single-strand RNA virus-like HCV and other flaviviruses [50, 51]. These viruses share a similar replication mechanism requiring an RNA-dependent RNA polymerase (RdRp). Elfiky used a molecular docking study to predict that some nucleosides such as Ribavirin, Remdesivir, Sofosbuvir, Galidesivir, and Tenofovir may have inhibitory activity against SARS-CoV-2 RdRp [52] or non-nucleosides [31, 32]. Significant efforts have been made in both vaccine research for the prevention of COVID-19, as well as

15
in the identification and utilization of novel or repurposed therapeutics for the management of those with symptomatic COVID-19. To date, eleven agents have received emergency use authorization (EUA) by the US Food and Drug Administration (US FDA) for the management of patients with COVID. This includes two nucleosides as antiviral agents, adenosine nucleoside analog remdesivir and β-D-N^4-hydroxycytidine (NHC, molnupiravir) [53]. In this context, all synthesized homonucleoside analogues (5a-h) were evaluated for their anti-SARS-CoV-2 activity against βCov/Belgium/GHB-03021/2020 in VeroE6 cell lines. All tested compounds showed no anti-SARS-CoV-2 activity, with EC50 >100µM. The coronavirus RdRp is a well-established drug target to treat coronavirus infections such as SARS, MERS, and now COVID-19. This polymerase shares similar catalytic mechanisms and displays active site conservation among different positive-sense RNA viruses, including coronaviruses [54]. Moreover, nucleotide analogues that inhibit polymerases are an important group of antiviral agents [55]. To prevent and slow SARS CoV-2 replication and disease progression, nucleoside analogues has been used. For instance, remdesivir and molnupiravir has been used to treat COVID-19. Remdesivir, a phosphoramidate prodrug, is converted into an adenosine triphosphate analogue inside virus-infected cells, which inhibits the RdRp of MERS-CoV, SARS-CoV, and SARS-CoV-2 [56]. It has been also demonstrated that molnupiravir can participate in viral replication and increase G to A and C to U substitution (Base pairing), introducing mutations in viral synthesis and inactivating the progeny viruses [57, 58]. As a result, the lack of activity of our compounds against SARS-CoV-2 can be explained by the fact that synthesized compounds are not recognized by the RdRp enzyme. Moreover, in contrast to molnupiravir and remdesivir the absence of the primary alcohol (which undergoes phosphorylation by the enzymes) in synthesized compounds could be a secondary reason for no activity.

**Annexin V7-AAD dual staining analysis**

Flow cytometry was carried out using annexin-V/7-AAD dual staining analysis to study the mechanism of cell death [59]. The HT-1080 and A-549 cell lines were treated with 20 µM of oxadiazole derivative 5e for 24 hours. The data is shown in Figure 2. The early apoptosis percentage increased from 0.06 to 1.02% for HT-1080 and from 1.47 to 11.62% for A-549 cell lines compared to the control. The rate of early apoptosis was enhanced by 17- and 8-fold in HT-10810 and A-549 lines, respectively. The late apoptosis rate was increased from 0.25 to 15.81% in HT-1080 cell lines and from 0.29 to 12.28% in A-549. There was also a significant increase (more than 60-fold) in the levels of total apoptosis in the DOX-treated cancer cell
lines. Thus, this product induces apoptosis, which is characterized by significant positive staining of annexin V.

Figure 2: Annexin V-FITC/7-AAD double staining for detection of apoptosis in HT-1080 and A549 cells after treatment with 20 µM of compound 5e as well as control (DMSO) for 24 hours. Compound 5e induces apoptosis.
Effect of compound 5e on Caspase 3/7 activity

The mechanism of apoptotic induction by the most potent compound 5e was investigated using caspase 3/7 activation assay in HT-1080 and A-549 cell lines. The proteolytic activity of caspase 3/7 was studied by the effect of compound 5e at 20μM for 24 hours. As presented in Figure 3, the rate of caspase-3/7 was increased from 6.38 and 4.55 to 19.03 and 16.2 in HT-1080 and A-549 cell lines, respectively. Thus, the percentage of caspase 3/7 was enhanced by 2.54- and 3.6-fold in HT-1080 and A-549 cell lines. On the other hand, there was a significant increase (more than 15-fold) in the levels of caspase-3/7 in DOX-treated cancer cell lines. Accordingly, these results were concordant with that of the flow cytometric analysis of apoptosis and revealed that compound 5e induced caspase 3/7 activation in fibrosarcoma and adenocarcinoma cancer cell lines.
Figure 3: Induction of caspase-3/7 activity in response to compound 5e. HT-1080 and A-549 Cells were treated with 20 µM of desired compound as well as DMSO (control) for 24 hours.

**Effect of Compound 5e on cell cycle distribution**

The effect of compound 5e on cell cycle arrest in HT-1080 and A-549 cell lines was determined by flow cytometry. The selected cells were treated with 20 µM of 5e and DMSO as control. The Analysis showed that compound 5e induces arrest in the G2M phase of the
cell cycle. Indeed, the product decreases the rate of G2M from 14.14% and 13.0 to 18.7% and 15.8% in HT-1080 and A-549 cell lines, respectively (Fig. 3). Meanwhile, there was a significant increase in the rate of G2M in DOX-treated HT-108 and A-549 cancer cell lines; from 14.4 and 13% to 26.8% and 18%, respectively.

![Flow cytometry analysis of cell cycle phase distribution in HT-1080 and A-549 cell lines after treatment of 20 µM of compound 5e in comparison with control for 24 hours.](image)

**Figure 4:** Flow cytometry analysis of cell cycle phase distribution in HT-1080 and A-549 cell lines after treatment of 20 µM of compound 5e in comparison with control for 24 hours.

**Molecular Docking**

Considering the docking study of oxadiazole derivatives as potent caspase-3 activator [35, 60, 61], we have continued exploring the apoptosis induction through the caspase activation
using in-silico molecular docking. Thus, compound 5e was docked into the active site of caspase-3 (PDB ID: 6CKZ). Prior to this, self-docking experiments were set-up to determine the validity of our docking protocol. The root-mean-square deviations (RMSD) between the predicted and the native poses were found to be 0.95 Å. These results indicated that the adopted docking protocol is acceptable for reproducing the native poses (<2 Å).

The docking results of the oxadiazole 5e, 5g, and 5h with caspase-3 are summarized in Figures 5-8 and table 2. The compounds 5e, 5g, and 5h could be docked into the active site of caspase-3 with the estimated free energies of binding of -7.8, -7.52, and -8.12 kcal/mol, respectively. Moreover, the estimated inhibition constant of compounds 5e, 5g, and 5h are 1.12, 3.09, and 1.12, respectively. Meanwhile, DOX has smaller binding energy (absolute value) than those of the investigated compounds. We could explain this by the fact that the DOX activates caspases by a different mechanism than the prepared products (it does not interact directly with caspase 3). Indeed, it was demonstrated that DOX-induced apoptosis is mainly initiated by oxidative DNA damage mediated by H₂O₂, leading to the ΔΨm increase and subsequent caspase-3 activation [62]. The 4 chlorophenyl moiety of product 5e was surrounded by a partially hydrophobic cavity (Figure 5). The three synthesized compounds form hydrogen bonds with the caspase-3. Other interactions were also observed, such as Pi-cation, Pi-sulfur, Pi-Pi T-shaped, and Pi-alkyl. We notice that all ligands interact with the key amino acids ARG207, HIS121, GLN161, and Tyr204 (Fig 6). These results are in line with the literature, which stated that these interactions are fundamental for caspase-3 activation [35, 61, 63-67]. As a result, the promising activity of compounds 5e, 5g, and 5h could be mediated by binding to the active sites of caspase-3.

Table 2: Binding energy and inhibition constant of compounds 5e, 5g, 5h, and DOX

| Compounds | Binding Energy (Kcal/mol) | Inhibition constant (µM) |
|-----------|---------------------------|--------------------------|
| 5e        | -8.12                     | 1.12                     |
| 5g        | -7.52                     | 3.09                     |
| 5h        | -8.12                     | 1.12                     |
| DOX       | -6.05                     | 37.5                     |
Figure 5: The position of ligand 5e in the hydrophobic cavity of caspase-3

Figure 6: 3D and 2D interactions of compound 5e with the amino acid residues of caspase-3
Figure 7: 2D interactions of compounds 5g and 5h with the amino acid residues of caspase-3

Figure 8: 2D interactions of DOX with the amino acid residues of caspase-3

Conclusion
A novel series of 5-fluorocytosine-1,3,4-oxadiazole conjugate derivatives were synthesized, fully characterized, and evaluated for their anticancer activities in HT-1080, A-549, MCF-7, and MDA-MB-23 cell lines. Among all synthesized derivatives, 5e showed promising cytotoxic activity against HT-1080 cancer cell lines, with an IC₅₀ value of 19.56 ± 1.62 µM. The mechanism of the anticancer activity on HT-1080 and A-549 was apoptotic by activation of caspase-3/7 with cell arrest at the G2M phase. Also, the molecular docking showed that compound 5e entered deep inside the binding site of caspase-3 by forming H-bond, carbon-hydrogen bond, Pi-cation, Pi-sulfur, Pi-Pi T-shaped, and Pi-alkyl type non-covalent interactions.

In summary, 5-fluorocytosine-1,3,4-oxadiazole hybrid derivatives were successfully repurposed in this discovery research paper to act as promising anticancer agents. The findings of this study open new avenues for future research to synthesize more potent derivatives with improved pharmacokinetic properties.

Declaration of Competing Interest

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

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CRediT authorship contribution statement

Az-eddine El Mansouri: Methodology, Writing – original draft, Software, Data curation. Saida Lachhab: Methodology, Writing – original draft, Data curation. Ali Oubella: Data curation, Formal analysis, Methodology. Mehdi Ahmad: Methodology, Writing – review & editing. Johan Neyts: Methodology, Writing – review & editing. Dirk Jochmans: Methodology, Writing – review & editing. Winston Chiu: Formal analysis. Laura Vangeel: Formal analysis. Steven De Jonghe: Formal analysis. Hamid Morjani: Data curation, Formal analysis, Methodology, Supervision, Writing – review & editing. Mustapha Ait Ali: Formal analysis, Supervision and Methodology. Mohamed Zahouily: Formal analysis, Supervision and Methodology. Yogesh sanghvi: Validation, Supervision, Writing – review & editing.
**H.B. Lazrek:** Conceptualization, Methodology, Project administration, Validation, Supervision, Writing – review & editing

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Graphical abstract

Cytotoxic Activity: 
IC₅₀ = 19.56 μM against HT-1080

Caspase-3/7 activation + Cell cycle arrest

Molecular docking

1,3,4-oxadiazole-5-fluorocytosine hybrid 5e

5-fluorocytosine