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Synthesis and antiproliferative activity of some novel amides of flufenamic acid and diclofenac

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ABSTRACT

*Coupling of the non-steroidal anti-inflammatory drugs (NSAIDs), flufenamic acid **2** and diclofenac **5** with the appropriate amino acid ester **6**, using dicyclohexylcarbdiimide (DCC), afforded amides **7-17**, respectively. The structures of the newly synthesized amides **7-17** were confirmed on the basis of their spectral data and X-ray single crystal analysis. All the synthesized compounds have been screened for anti-proliferative activity against A549 lung adenocarcinoma and HT-29 colon cancer cells. Compound **17** showed a good anti-proliferative activity IC_{50} = 25.82 µM compared with the chemotherapeutic agent 5-fluorouracil IC_{50}= 18 µM. Furthermore, compound **8** was more potent than the reference drug with IC_{50}= 15.4µM and cell cycle blockade activity at G_0 and S-phase. Compounds **11**, **12**, **13** and **17** were more potent than the reference drug dichlorofluorescein diacetate (DCF) IC_{50}= 17.81 µM, while the IC_{50} for these compound ranges from 6.46-13.99 µM.*

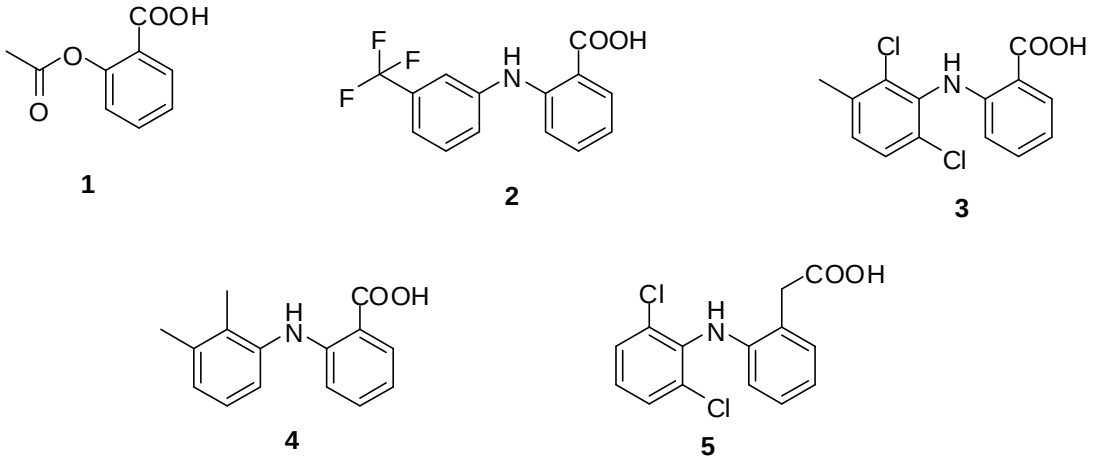
Keywords: Flufenamic; Diclofenac; Amino acid ester; Cancer: Colorectal cancer.

INTRODUCTION

Non steroidal antiinflammatory drugs NSAIDs are among the most frequently prescribed drugs in modern medicine. They are very effective in the alleviation of pain, fever and inflammation [1]. Millions of patients worldwide have found pain relief in their use since the discovery of the soothing properties of willow bark more than 3,500 years ago [2]. NSAIDs exert their antiinflammatory effect through inhibition of cyclooxygenase, an enzyme that catalyzes the transformation of arachidonic acid to prostaglandins and thromboxanes [3]. The global burden of cancer continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing behaviors, particularly smoking in economically developing countries. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world [4]. The first report of a protective role for aspirin **1** (Figure 1) against cancer development appeared in 1988, documenting a negative association with colorectal cancer. Subsequently, a very large number of studies have been carried out demonstrating similar effects in the colon and/or rectum. Beneficial effects have also been described for other organs [5]. In terms of their antineoplastic activity, in vitro studies have shown that the mechanism is mainly due to the induction of apoptosis and prevention of cell proliferation [6]. Other mechanistic studies suggest that a COX-independent or off-target effect, possibly involving phosphodiesterase-5 (PDE-5) inhibition and cyclic guanosine monophosphate (cGMP) elevation to induce apoptosis may contribute to their antineoplastic properties [7]. Fenamic acids (flufenamic **2**, meclofenamic **3** and mefenamic acids **4**) (Figure 1) are known to have high antiproliferative activity, also they show inhibition of the stimulated calcium uptake in cells, suggesting that they give a signal for

apoptosis [8]. Diclofenac **5** (Figure 1) on the other hand, exhibit high potency against HT29 colorectal adenoma cell lines [9].

Figure 1. Structure of drugs 1-5



Recently, many studies have revealed that amide and ester modification of the carboxylic group of the existing NSAIDs impart an enhanced potency and safety on these drugs as antiproliferative agents as well as utility for treating malignant disease if combined with chemotherapy, this may be due to an enhanced cellular uptake as well as lipophilicity of these derivatives [10-14].

Literature survey revealed that many efforts had been made to synthesize amino acid ester, glycolamide ester, and amide prodrugs using various amines, but few attempts were made to develop amide prodrugs using amino acids [15]. The salient features of the usefulness of conjugation of amino acids with NSAIDs have been reported [16]. In the light of previous data and in continuation of our interest in development of safe NSAIDs derivatives suitable for use as chemopreventive agent, a series of flufenamic acid **2** and diclofenac **5** amides of amino acid esters were synthesized and their antiproliferative activities have been investigated against colon and lung cancer human cell lines.

MATERIALS AND METHODS

3.1. General

Flufenamic acid, diclofenac and amino acid ester hydrochloride were obtained from Sigma-aldrich, the other reagents and solvents used were of analytical grade, the reactions were monitored by TLC on precoated silica G plates using uv lamp. Melting points were determined on a Gallenkamp melting point apparatus, and are uncorrected. Infrared (IR) Spectra were recorded as KBr discs using the Perkin Elmer FT–IR Spectrum BX apparatus located at the Research Center, College of Pharmacy, King Saud University (Riyadh, Saudi Arabia). NMR Spectra were scanned in DMSO-d_6 on a Bruker NMR spectrometer operating at 500 MHz for ^{1}H and 125.76 MHz for ^{13}C at the Research Center, College of Pharmacy, King Saud University (Riyadh, Saudi Arabia). Chemical shifts are expressed in δ-values (ppm) relative to TMS as an internal standard. Coupling constants (J) are expressed in Hz. D_2O was added to confirm the exchangeable protons. Mass spectra were measured on Agilent Triple Quadrupole 6410 QQQ LC/MS with ESI (Electrospray ionization) source.

3.2. General procedure for synthesis of compounds **7-17**.

Flufenamic acid or diclofenac (0.01 mol) was dissolved in dichloromethane (DCM) (20 mL) followed by addition of dicyclohexylcarbodiimide (DCC) (0.015 mol), the reaction mixture was stirred for 30 min and a mixture of appropriate amino acid hydrochloride (0.02 mol) and triethylamine (TEA, 0.2 mL) in DCM (10 mL) was added drop-wise. The reaction mixture was stirred at 0^{o}C initially for 2 hrs followed by stirring at room temperature for overnight, the precipitated dicyclohexyl urea (DHU) was filtered off; the solvent was distilled off under reduced pressure. Ethyl acetate (10 mL) was added to the dried product to remove any remaining DHU. The ethyl acetate layer was washed with aqueous solution of sodium bicarbonate (10%) and then with distilled water to remove triethylamine hydrochloride and any traces of alkali. Ethyl acetate layer was dried over anhydrous magnesium sulphate, filtered off and the filtrate was distilled off under reduced pressure to obtain the crude product, which was then purified by column chromatography using ethyl acetate:hexanes (9:1), then they were recrystallized from the appropriate solvent. (for yield and melting points see Table 1).

32.1. Ethyl 2-(2-(3-(trifluoromethyl) phenylamino) benzamido) acetate **7**.
Compound **7** was obtained as white powder. IR (KBr): ῡ/cm^{-1} 3245 (NH), 1742 (C=O, ester), 1632 (C=O, amide). ^{1}H NMR (DMSO-d6): δ 1.18 (t, 3H, J = 7 Hz, -OCH$_2$C**H$_3$**), 3.99 (d, 2H, J = 6 Hz, C**H$_2$**CO), 4.09-4.13 (q, 2H, J = 7 Hz, OCH$_2$C**H$_3$**), 6.97 (t, 1H, J = 8 Hz, Ar-**H**), , 7.22 (d, 1H, J = 7.5 Hz, Ar**H**), 7.35 (d, 1H, J = 8 Hz, Ar**H**), 7.40-7.42 (m, 3H, Ar**H**), 7.48 (t, 1H, J = 8 Hz, Ar**H**), 7.72 (d, 1H, J = 8 Hz, Ar**H**), 9.03 (t, 1H, J = 6 Hz, -CON**H**, D$_2$O exchangeable), 9.6 (s, 1H, ArN**H**Ar, D$_2$O exchangeable). ^{13}C NMR (DMSO-d6): δ 13.93 (-OCH$_2$**C**H$_3$), 41.17 (NH**C**H2-), 60.50 (-O**C**H$_2$CH$_3$), 114.38, 116.44, 117.30, 119.71, 120.02, 120.81, 121.84, 122.98 (Ar-**C**), 125.14 (-**C**F$_3$), 128.94, 130.39, 132.16, 142.78, 142.81 (Ar-**C**), 168.92 (C=O amide), 169.70 (C=O ester). MS-ESI: m/z 367.12(M+1).

3.2.2. (R)-Methyl2-(2-(3-(trifluoromethyl)phenylamino)benzamido)propanoate **8**.
Compound **8** was obtained as colorless oil. IR (CHCl$_3$): ῡ/cm^{-1} 3312 (NH), 1740 (C=O, ester), 1636(C=O, amide). ^{1}H NMR (DMSO-d6): δ 1.41 (d, 3H, J = 7 Hz, -CH-C**H$_3$**) 3.66 (s, 3H, -OC**H$_3$**), 4.51-4.54 (m, 1H, -C**H**NH), 6.97 (t, 1H, J = 8 Hz, Ar-**H**), 7.22 (d, 1H, J = 7.5 Hz, Ar**H**), 7.35 (d, 1H, J = 8Hz, Ar**H**), 7.39-7.48 (m, 3H, Ar**H**), 7.48 (t, 1H, J = 8 Hz, Ar**H**), 7.81 (d, 1H, J = 8 Hz, Ar**H**), 8.93 (d, 1H, J = 6.5 Hz, -CON**H**, D$_2$O exchangeable), 9.6 (s, 1H, ArN**H**Ar, D$_2$O exchangeable). ^{13}C NMR (DMSO-d6): δ 16.45 (-CH-**C**H$_3$), 48.13 (-**C**HNH-), 51.75 (-O**C**H$_3$), 114.34, 116.44, 117.15, 119.60, 120.29, 120.81, 121.71, 122.97 (Ar-**C**), 125.14 (-**C**F$_3$), 129.34, 130.39, 132.00, 142.73, 142.91 (Ar-**C**), 168.44 (C=O amide), 173.00 (C=O ester). MS-ESI: m/z 367.33 (M+1).

3.2.3. Ethyl 3-(2-(3-(trifluoromethyl)phenylamino)benzamido)propanoate **9**.
Compound **9** was obtained as colourless oil. IR (CHCl$_3$): ῡ/cm^{-1} 3246 (NH), 1727 (C=O, ester), 1627(C=O, amide). ^{1}H NMR (DMSO-d6): δ 1.15 (t, 3H, J = 7 Hz, O-CH$_2$-C**H$_3$**), 2.56 (t, 2H, J = 7 Hz, -C**H$_2$**CO-), 3.48-3.52 (q, 2H, J = 7 Hz, -NHC**H$_2$**-), 4.03-4.07 (q, 2H, J = 7 Hz, OC**H$_2$**CH$_3$), 6.94 (t, 1H, J =8 Hz, Ar-**H**), 7.19 (d, 1H, J = 8 Hz, Ar**H**), 7.35 (d, 1H, J= 8, Ar**H**), 7.33-7.38 (m, 3H, Ar**H**), 7.46 (t, 1H, J=8, Ar**H**), 7.65-7.67 (dd, 1H, J = 8,2 Hz, Ar**H**), 8.65 (t, 1H, J = 5.5 Hz, -CON**H**, D$_2$O exchangeable), 9.63 (s, 1H, ArN**H**Ar, D$_2$O exchangeable). ^{13}C NMR (DMSO-d6): δ 13.87 (O-CH$_2$-**C**H$_3$), 33.54 (-**C**H$_2$CO-), 35.28 (-NH**C**H$_2$-), 59.86 (-O**C**H$_2$CH$_3$), 114.00, 116.73, 116.95, 119.79, 120.83, 121.41, 122.99 (Ar-**C**), 125.16 (-**C**F$_3$), 128.94, 130.27, 131.71, 142.37, 143.10 (Ar-**C**), 168.44 (C=O amide), 171.22 (C=O ester). MS-ESI: m/z 381.3 (M+1).

3.2.4. (S)-Dimethyl 2-(2-(3-(trifluoromethyl)phenylamino)benzamido)succinate **10.**
Compound **10** was obtained as yellow powder. IR (KBr): ῡ/cm^{-1} 3348 (NH), 1749 (C=O, ester), 1641(C=O, amide). ^{1}H NMR (DMSO-d6): δ 2.80-2.85 (dd, 1H, J = 7.5 Hz, 16 -C**H$_2$**CO), 2.89-2.94 (dd, 1H, J = 6 Hz, 16, -C**H$_2$**CO), 3.60 (s, 3H, -CH$_2$COOC**H$_3$**), 3.64 (s, 3H, -CHCOOC**H$_3$**), 4.84-4.85 (m, 1H, -C**H**NH),), 7.00 (t, 1H, J = 8 Hz, Ar-**H**), 7.23 (d, 1H, J = 8 Hz, Ar**H**), 7.34 (d, 1H, J = 8 Hz, Ar**H**), 7.40-7.43 (m, 3H, Ar**H**), 7.46 (t, 1H, J = 8 Hz, Ar**H**), 7.47 (d, 1H, J= 8, 2, Ar**H**), 9.01 (d, 1H, J = 8 Hz, -CON**H**, D$_2$O exchangeable), 9.44 (s, 1H, ArN**H**Ar, D$_2$O exchangeable). ^{13}C NMR (DMSO-d6): δ 35.19 (**C**H$_2$CO), 48.97 (-NH**C**H-), 51.62 (CH$_2$COO**C**H$_3$), 52.24 (CHCOO**C**H$_3$), 114.32, 116.93, 117.23, 119.96, 120.46, 121.71, 123.01 (Ar-**C**), 125.17 (-**C**F$_3$), 129.23, 130.26, 132.30, 142.57, 142.99 (Ar-**C**), 168.18 (C=O amide), 170.48 (-CH$_2$**C**OOCH$_3$), 171.05 (-CH**C**OOC**H$_3$**). MS-ESI: m/z 424.3 (M$^+$).

3.2.5. Ethyl 2-(2-(2-(2,6-dichlorophenylamino)phenyl)acetamido)acetate **11**.
Compound **11** was obtained as white crystals. IR (KBr): ῡ/cm^{-1} 3245 (NH), 1742 (C=O, ester), 1632 (C=O, amide). ^{1}H NMR (DMSO-d$_6$): δ 1.16 (t, 3H, J = 7 Hz -OCH$_2$C**H$_3$**), 3.66 (s, 2H, ArC**H$_2$**-) 3.89 (d, 2H, J = 6 Hz, -NH-C**H$_2$**-), 4.07-4.11 (q, 2H, J = 7 Hz, -O-C**H$_2$**CH$_3$), 6.29 (d, 1H, J = 8 Hz, Ar**H**), 6.86 (t, 1H, J = 7.5 Hz, Ar**H**), 7.05 (t, 1H, J =7.5 Hz, Ar**H**), 7.16 (t, 1H, J = 8 Hz, Ar**H**), 7.22-7.24 (dd, 1H, J = 1.5 Hz, 7.5 Ar**H**), 7.50 (d, 2H, J = 8 Hz, Ar**H**), 8.09 (s, 1H, ArN**H**Ar, D$_2$O exchangeable), 8.80 (t, 1H, J = 6 Hz, -CON**H**-, D$_2$O exchangeable). ^{13}C NMR (DMSO-d$_6$): δ 13.94 (OCH$_2$**C**H$_3$), 38.98 (Ar**C**H$_2$-), 40.80 (-NH-**C**H$_2$-), 60.46 (-O**C**H$_2$CH$_3$), 115.98, 120.68, 125.09, 125.13, 127.24, 129.13, 129.51, 130.40, 137.15, 142.90, (Ar-**C**), 169.60 (C=O amide), 172.04 (C=O ester). MS-ESI: m/z 382.2 (M+1). MS-EI: m/z (%) 279 (21), 277 (33), 244 (16), 242 (47), 216 (32), 214 (100), 179 (14), 162 (15), 151 (10), 102 (25), 86 (15), 59 (17).

3.2.6. (R)-Methyl2-(2-(2-(2,6-dichlorophenylamino)phenyl)acetamido)propanoate **12**.
Compound **12** was obtained as white powder. (KBr): ῡ/cm^{-1} 3312 (NH), 1740 (C=O, ester), 1636 (C=O, amide). ^{1}H NMR (DMSO-d$_6$): δ 1.31 (d, 3H, J = 7 Hz, -C**H$_3$**CH-), 3.39 (s, 2H, ArC**H$_2$**-) 3.62 (s, 3H, -OC**H$_3$**), 4.32-4.37 (m, 1H, -NHC**H**-), 6.29 (d, 1H, J = 8Hz, Ar**H**), 6.86 (t, 1H, J = 7.5 Hz, Ar**H**), 7.04 (t, 1H, J = 7.5 Hz, Ar**H**), 7.15 (t, 1H, J = 8.5 Hz, Ar**H**), 7.22-7.24 (dd, 1H, J =1.5, 8 Hz, Ar**H**), 7.50 (d, 2H, J = 8 Hz, Ar**H**), 8.06 (s, 1H, ArN**H**Ar, D$_2$O exchangeable), 8.80 (d, 1H, J = 7 Hz, -CON**H**-, D$_2$O exchangeable). ^{13}C NMR (DMSO-d$_6$): δ 17.03(**C**H$_3$CH-), 38.93 (Ar**C**H$_2$-), 47.63 (-NH-**C**H-), 51.87 (-O**C**H$_3$), 115.92, 120.65, 125.09, 127.22, 129.11, 129.58, 130.42, 137.15, 142.91, (Ar-**C**), 171.34 (C=O amide), 172.86 (C=O ester). MS-ESI: m/z 382.2 (M+1). MS-EI: m/z (%) 279 (14),

277 (30), 244(12), 242 (12), 214 (100), 124 (16), 122 (10), 151 (14), 132 (14), 98 (7), 77 (15), 72 (64), 59 (29), 55 (56), 43 (62), 41 (45).

3.2.7. (S)-Methyl2-(2-(2-(2,6-dichlorophenylamino)phenyl)acetamido)propanoate **13**.
Compound **13** was obtained as white powder. IR (KBr): ῡ/cm^{-1} 3312(NH), 1739 (C=O, ester), 1636 (C=O, amide). ^{1}H NMR (DMSO-d6); δ 1.31 (d, 3H, J = 7Hz, -C**H$_3$**CH-), 3.39 (s, 2H, ArC**H$_2$**-) 3.62 (s, 3H, -OC**H$_3$**), 4.32-4.37 (m, 1H, -NHC**H**-), 6.29 (d, 1H, J = 8 Hz, Ar**H**), 6.86 (t, 1H, J = 7.5 Hz, Ar**H**), 7.04 (t, 1H, J =7.5 Hz, Ar**H**), 7.15 (t, 1H, J = 8.5 Hz, Ar**H**), 7.22-7.24 (dd, 1H, J = 1.5, 8 Hz, Ar**H**), 7.50 (d, 2H, J = 8 Hz, Ar**H**), 8.06 (s, 1H, ArN**H**Ar, D_2O exchangeable), 8.80 (d, 1H, J = 7 Hz, -CON**H**-, D_2O exchangeable). ^{13}C NMR ($CDCl_3$): δ 18.53(**C**H_3CH-), 40.80 (Ar**C**H_2-), 48.23 (-NH-**C**H-), 52.53 (-O**C**H_3), 117.72, 121.65, 124.16, 124.53, 128.01, 128.85, 129.95, 130.68, 137.72, 142.98 (Ar-**C**), 171.13 (C=O amide), 173.43 (C=O ester). MS-ESI: m/z 382.2 (M+1). MS-EI: m/z (%) 279 (25), 277 (35), 244 (16), 242 (45), 216 (34), 215 (27), 214 (100), 179 (14), 132 (16), 124 (16), 72 (48), 59 (11), 55 (17).

3.2.8. Ethyl 3-(2-(2-(2,6-dichlorophenylamino)phenyl)acetamido)propanoate **14**.
Compound **14** was obtained as white powder. IR (KBr): ῡ/cm^{-1} 3246 (NH), 1727 (C=O, ester), 1627 (C=O, amide). ^{1}H NMR (DMSO-d_6): δ 1.16 (t, 3H, J = 7 Hz, -O-CH_2-C**H$_3$**), 2.48 (t, 2H, J = 7 Hz, -NHCH_2C**H$_2$**-) 3.30-3.34 (q, 2H, J = 6 Hz , -NHC**H$_2$**CH_2-), 3.58 (s, 2H, ArC**H$_2$**-), 4.02-4.07 (q, 2H, J = 7 Hz, O-C**H$_2$**CH_3), 6.28 (d, 1H, J = 8 AHz, r**H**), 6.84 (t, 1H, J = 7.5 Hz, Ar**H**), 7.04 (t, 1H, J = 8 Hz, Ar**H**), 7.15 (t, 1H, J = 8.5 Hz, Ar**H**), 7.17-7.19 (dd, 1H, J = 1.5, 8 Hz, Ar**H**), 7.50 (d, 2H, J = 8 Hz, Ar**H**), 8.32 (s, 1H, ArN**H**Ar , D_2O exchangeable), 8.47 (t, 1H, J = 6 Hz, -CON**H**-, D_2O exchangeable). ^{13}C NMR (DMSO-d_6): δ 13.99 (-OCH_2**C**H_3), 33.63 (-NHCH_2**C**H_2-), 34.89 (Ar**C**H_2-), 39.01 (-NH**C**H_2-), 59.92 (-O**C**H_2-CH_3), 115.89, 120.58, 124.99, 125.33, 127.16, 129.13, 129.44, 130.36, 137.16, 142.93 (Ar-**C**), 171.20 (C=O amide), 171.65 (C=O ester). MS-ESI: m/z 395.2 (M^+). MS-EI: m/z (%) 380 (6), 279 (23), 277 (33), 244 (19), 242 (48), 216 (35), 215 (25), 214 (100), 179 (15), 151 (11), 104 (13), 89 (10), 77 (9), 44 (54).

3.2.9. (R)-Methyl2-(2-(2-(2,6-dichlorophenylamino)phenyl)acetamido)-3methyl butanoate **15**.
Compound **15** was obtained as white powder. IR (KBr): ῡ/cm^{-1} 3299 (NH), 1742 (C=O, ester), 1644 (C=O, amide). ^{1}H NMR (DMSO-d_6): δ 0.85 (d, 3H, J = 7 Hz, one of (C**H$_3$**)$_2$CH-), 0.89 (d, 3H, J = 7 Hz, one of (C**H$_3$**)$_2$CH-), 2.03-2.09 (m, 1H, $(CH_3)_2$C**H**-), 3.64 (s, 3H, -OC**H$_3$**), 3.68 (d, 1H, J = 13.5 Hz, ArC**H$_2$**-), 3.77 (d, 1H, J = 13.5 Hz, ArC**H$_2$**-), 4.27 (t, 1H, J = 7 Hz, -NHC**H**-), 6.29 (d, 1H, J = 8 Hz, Ar**H**), 6.84 (t, 1H, J = 7.5 Hz, Ar**H**), 7.03 (t, 1H, J = 8 Hz, Ar**H**), 7.13 (t, 1H, J = 8 Hz, Ar**H**), 7.17-7.19 (dd, 1H, J = 1.5, 7.5 Hz, Ar**H**), 7.50 (d, 2H, J = 8 Hz, Ar**H**), 8.09 (s, 1H, ArN**H**Ar, D_2O exchangeable), 8.65 (d, 1H, J = 8 Hz, -CON**H**-, D_2O exchangeable). ^{13}C NMR (DMSO-d_6): δ 18.10 (one of (**C**H_3)$_2$CH-), 18.82 (one of (**C**H_3)$_2$CH-), 30.11 $(CH_3)_2$**C**H-), 35.05 (Ar**C**H_2-), 51.32 (-O**C**H_3), 59.69 (-NH**C**H-), 115.87, 120.61, 124.53, 125.30, 127.13, 129.07, 129.57, 130.37, 137.16, 142.88 (Ar-**C**), 171.20 (C=O amide), 173.12 (C=O ester). MS-ESI: m/z 409.3 (M^+). MS-EI: m/z (%) 279 (22), 277 (40), 244 (19), 242 (62), 216 (29), 215 (15), 214 (100), 179 (34), 178 (19), 151 (20), 106 (20), 89 (30), 78 (33), 76 (15), 75 (18), 63 (25), 52 (20), 51 (30).

3.2.10. (S)-Ethyl 2-(2-(2-(2,6-dichlorophenylamino)phenyl) acetamido)-4-methyl pentanoate **16**.
Compound **16** was obtained as white powder. IR (KBr): ῡ/cm^{-1} 3322 (NH), 1742 (C=O, ester), 1639 (C=O, amide). ^{1}H NMR (DMSO-d_6): δ 0.82 (d, 3H, J = 6.5 Hz, one of (C**H$_3$**)$_2$CH-), 0.88 (d, 3H, J = 6.5 Hz, one of (C**H$_3$**)$_2$CH-), 1.13 (t, 3H, J = 7 Hz,-OCH_2C**H$_3$**), 1.50-1.65 (m, 3H, $(CH_3)_2$C**H**-) and $(CH_3)_2$CH-C**H$_2$**-), 3.62 (d, 1H, J = 14 Hz, ArC**H$_2$**-), 3.68 (d, 1H, J = 13.5 Hz, ArC**H$_2$**-), 4.03-4.06 (q, 2H, J = 7, OC**H$_2$**CH_3), 4.29-4.32 (q, 1H, J = 7.5 Hz, -NHC**H**-), 6.28 (d, 1H, J=8 Ar**H**), 6.85 (t, 1H, J = 7.5 Hz, Ar**H**), 7.04 (t, 1H, J = 8 Hz, Ar**H**), 7.16 (t, 1H, J = 8 Hz, Ar**H**), 7.22 (d, 1H, J = 7.5 Hz, Ar**H**), 7.50 (d, 2H, J = 8 Hz, Ar**H**), 8.03 (s, 1H, ArN**H**Ar, D_2O exchangeable), 8.71 (d, 1H, J = 7.5 Hz, -CON**H**-, D_2O exchangeable). ^{13}C NMR (DMSO-d_6): δ 13.89 (-OCH_2**C**H_3), 21.32 (one of (**C**H_3)$_2$CH-CH_2-), 22.61 (one of (**C**H_3)$_2$CH-CH_2), 24.24 $(CH_3)_2$**C**H-), 38.92 (Ar**C**H_2-), 50.51 (-NH**C**H-), 60.43 (-O**C**H_2CH_3), 115.84, 120.59, 125.12, 127.18, 129.12, 129.60, 130.36, 137.14, 142.87 (Ar-**C**), 171.56 (C=O amide), 172.24 (C=O ester). MS-ESI: m/z 437.3 (M+1).

3.2.11. (S)-Dimethyl 2-(2-(2-(2,6-dichlorophenylamino)phenyl)acetamido)succinate **17**.
Compound **17** was obtained as white crystals. IR (KBr): ῡ/cm^{-1} 3348 (NH), 1749 (C=O, ester), 1641(C=O, amide). ^{1}H NMR (DMSO-d6): δ 2.75-2.80 (dd, 1H, J = 4.5, 18 Hz, -C**H$_2$**CO), 2.80-2.86 (dd, 1H, J = 4.5, 18 Hz, -C**H$_2$**CO), 3.35 (s, 3H, -CH_2COOC**H$_3$**), 3.62 (s, 3H, -CHCOOC**H$_3$**), 3.64 (s, 2H, ArC**H$_2$**-) 4.69-4.73 (q, 1H, J = 6.5 Hz, -C**H**NH), 6.29 (d, 1H, J = 8 Hz, Ar**H**), 6.85 (t, 1H, J = 7.5 Hz, Ar**H**), 7.04 (t, 1H, J = 8 Hz, Ar**H**), 7.15 (t, 1H, J = 8 Hz, Ar**H**), 7.20 (d, 1H, J = 7.5 Hz, Ar**H**), 7.51 (d, 2H, J = 8 Hz, Ar**H**), 7.93 (s, 1H, ArN**H**Ar, D_2O exchangeable), 8.87 (d, 1H, J = 8 Hz, -CON**H**-, D_2O exchangeable). ^{13}C NMR (DMSO-d6): δ 35.61 (Ar**C**H_2) 38.79 (**C**H_2CO), 48.54 (-NH**C**H-), 51.61, (CH_2COO**C**H_3), 52.20, (CHCOO**C**H_3), 115.81, 120.61, 124.90, 125.23, 127.24, 129.13,

129.72, 130.39, 137.12, 142.87 (Ar-**C**), 170.32 (-**C**ONH), 170.87 (CH_2**C**$OOCH_3$), 171.42 (-CH**C**$OOCH_3$). MS-ESI: m/z 439.3 (M^+).

3.2.12. N-Cyclohexyl-N-(cyclohexylcarbamoyl)-2-(3-(trifluoromethyl)phenylamino)benzamide **18**.
Compound **18** was obtained as yellow powder m.p (106-110 **°C**). IR (KBr): ῡ/cm^{-1}3254.2 (ArNHAr), 3042 (Ar-H), 1652 (C=O). ^{1}H NMR (DMSO-d6): δ 1.02-1.08 (m, 8H), 1.15-1.20 (m, 6H), 1.40-1.47 (m, 2H), 1.65-1.73 (m, 4H), 1.88-1.90 (m, 2H), 6.33 (d, 1H, J = 8 Hz, -CONH), 6.87-7.79 (8H, ArH), 8.52 (s, ArN**H**Ar). ^{13}C NMR (DMSO-d6): δ 18.46, 23.92, 24.31, 25.10, 25.21, 30.36 (Alifatic Carbons), 34.45, 37.6 (Alifatic Carbons), 49.81 (-**C**HNH-), 56.24 (-**C**H-N-), 114.00, 116.73, 116.95, 119.79, 120.83, 121.41, 122.99 (Ar-**C**), 125.16 (-**C**F_3), 128.94, 130.27, 131.71, 142.37, 143.10 (Ar-**C**), 153.7 (-NH**C**O-N-), 169.96 (**C**ON-).

3.2.13.N-Cyclohexyl-N- (cyclohexylcarbamoyl) – 2 - (2-(2,6dichlorophenylamino) phenyl) acetamide **19**.
Compound **19** was obtained as white powder m.p (158-160 **°C**). IR (KBr): ῡ/cm^{-1}: 3258.7 (ArNHAr), 3054 (Ar-H), 1650 (C=O), 770 and 741 (C-Cl). ^{1}H NMR (DMSO-d6): δ 1.02-1.08 (m, 8H), 1.15-1.20 (m, 6H), 1.40-1.47 (m, 2H), 1.65-1.73 (m, 4H), 1.88-1.90 (m, 2H), 3.75 (s, 2H, ArC**H**$_2$-), 6.33 (d, 1H, J= 8Hz, -CONH), 6.87-7.79 (7H, ArH), 8.56 (s, ArN**H**Ar). ^{13}C NMR (DMSO-d6): δ 18.49, 23.97, 24.34, 25.06, 25.2, 30.36 (Alifatic Carbons), 31.68 (Ar**C**H_2), 34.45, 37.6 (Alifatic Carbons), 49.75 (-**C**HNH-), 56 (-**C**H-N-), 116.45, 120.90, 124.8, 125.12, 127.36, 129.13, 129.19, 130.86, 137.26, 143.04 (Ar-C), 153.14 (-NH**C**O-N-), 168.76 (**C**ON-).

3.2.14. 1-(2,6-Dichlorophenyl)indolin-2-one **20**.
Compound **20** was obtained as white powder m.p (115-117 **°C**). IR (KBr): ῡ/cm^{-1}: 1731 (C=O lactam), 1612 (CN), 782 and 749 (C-Cl). ^{1}H NMR (DMSO-d6): δ 3.87 (s, 2H), 6.38 (d, 1H, J = 7.63 Hz), 7.08 (dt, 1H, J = 7.67 Hz and 1.05 Hz), 7,20 (dt, 1H, J = 7.67 Hz and 1.05 Hz), 7,38 (d, 1H, J = 7.67 Hz), 7,74 (d, 2H, J = 8.13 Hz), 7.60 (dd, 1H, J = 8.13 Hz). ^{13}C NMR δ (DMSO-d6): 35.05 (-**C**H_2), 108.47, 122.80, 124.57, 124.96, 127.78, 129.35, 129.86, 131.88, 134.42, 142.77 (Ar-**C**), 173.19 (C=O). MS-ESI: m/z 278.

4.1. Biological investigations
4.1.1. Antiproliferative activity against Human colon adenocarcinoma HT-29
4.1.1.1. Cell Line and WST-1 Cell Proliferation Assay
HT-29 colon adenocarcinoma cell line was purchased from the American Type Culture Collection. Cells were maintained in DMEM (Sigma), supplemented with 10% FBS (Lonza), 100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mmol/L L-glutamine (Sigma) and seeded into 96-well plates at 4 x10^3 cells /well and incubated overnight. The medium was replaced with fresh one that containing the desired concentrations of the compounds. After 72 h, 10 µl of the WST-1 reagent was added to each well and the plate was re incubated for 4 h at 37°C. The amount of formazan was quantified using ELISA reader at 450 nm [18].

4.1.2. Flow Cytometric analysis of cellular DNA content
Cells (2 x 10^6) were fixed in 1ml ethanol (70%) for 60min at room temperature. Harvested cells were resuspended in 1ml Na citrate (50 mM) containing 250µg RNase A and incubated at 50°C for 60min. Then the cells were resuspended in the same buffer containing 4µg propidium iodine (PI) and incubated for 30min before being analyzed by flow cytomerty (Becton Dickinson, San Jose, CA, USA). The percentage of cells in various cell cycle phases was determined by using Cell Quest Pro software (Becton Dickinson) [19].

4.1.3. Measurement of annexin V binding by flow cytometry
It has been reported that, the loss of phospholipid asymmetry of the plasma membrane is an early event of apoptosis. The annexin V binds to negatively charged phospholipids, like phosphatidylserine. During apoptosis, the cells react to annexin V once chromatin condenses but before the plasma membrane loses its ability to exclude propidium iodide (PI). Hence it is possible to detect non-apoptotic live cells, early apoptotic cells and late apoptotic or necrotic cells, by staining cells with a combination of fluorescein isothiocyanate (FITC) annexin V and PI, annexin-V staining was performed by using Vybrant Apoptosis Assay Kit # 2(Molecular Probe) following the manufacturer's recommendations. Annexin-V stained cells were analyzed by flow cytometry, measuring the fluorescence emission at 530 and less than 575 nm [20].

4.1.4. Antiproliferative activity against Human lung adenocarcinoma A549
4.1.4.1. Cells and Reagents
Human lung adenocarcinoma A549 cells were grown in RPMI medium (supplemented with 10% bovine serum, 1% penicillin-streptomycin, and 1%L-glutamate (HyClone Laboratories) at 37^{o}C in a humified chamber with 5% CO_2.

4.1.4.2. In vitro Cell proliferation or cytotoxicity) assay

The cytotoxic activity was measured in vitro for the newly synthesized compounds with a rapid colorimetric assay using MTT and compared with the untreated controls. This assay is based on the metabolic reduction of soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by mitochondrial enzyme activity of viable tumor cells, into an insoluble colored formazan product, which can be measured spectrophotometrically [21]. The cells were plated in flat bottom 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the compound(s) to allow attachment of cell to the wall of the plate. The test compounds were dissolved in dimethyl sulfoxide (DMSO), different concentrations of the compound under test (5, 12.5, 25, 50 µg) were added to the cell monolayer and the cells treated only with solvent were considered as negative control and the reference drug 2′,7′-dichlorofluorescein diacetate (DCF) was used as positive control. The final concentration of DMSO did not exceed 0.2%. Triplicate wells were prepared for each individual concentration. Treated cells were incubated for 48 h at 37°C and in atmosphere of 5% CO_2. The cells were then treated with MTT reagent (20 µl/well) for 4 h at 37°C and then MTT detergent (100 µl) was added to each well to dissolve the formazan crystals. The optical density (OD) was recorded at 490nm in a microplate reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for cancer cell line after the specified time. The molar concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in Table 1

RESULTS AND DISCUSSION

2.1. Chemistry

The target derivatives were obtained by coupling of flufenamic acid **2** and diclofenac **5** with the appropriate amino acid ester **6** using dicyclohexylcarbdiimide (DCC) in dicholoromethane (DCM) (Scheme 1, Table 1).

Scheme 1. Synthesis of the target amide derivatives 7-17

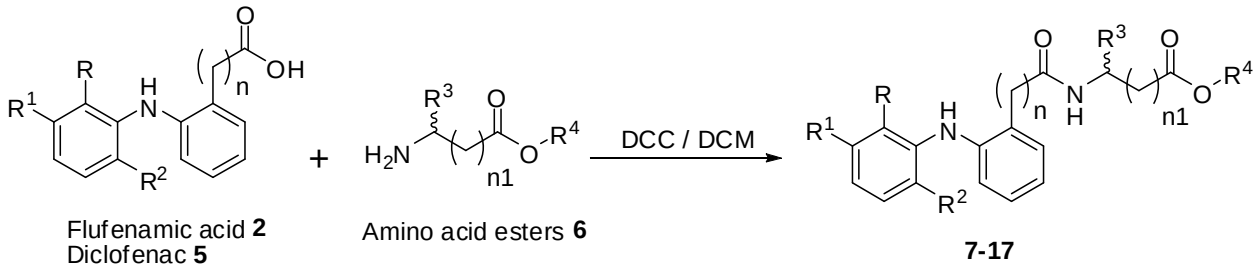


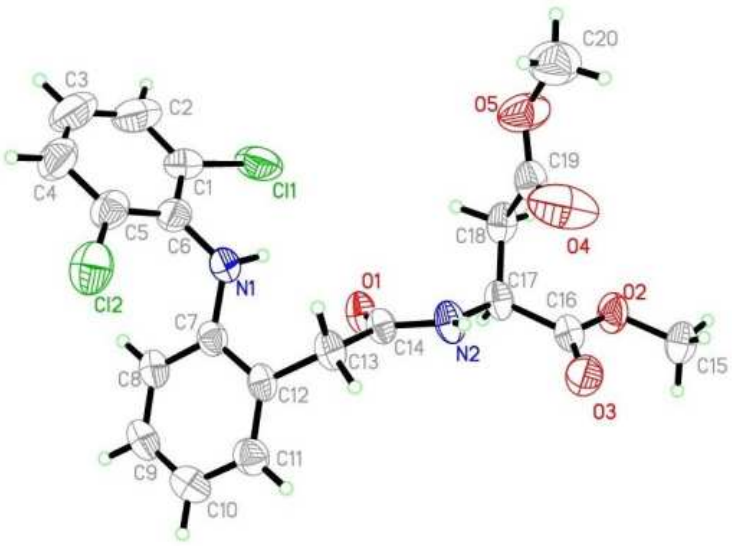
Table 1. Structure, M.P., yield and Clog P of compounds 7-17

Cmpd	R	R^1	R^2	R^3	R^4	n	n1	Amino acid ester (6)	M.P. (°C)	Yield	CloP
7	H	$-CF_3$	H	H	Et	0	0	Ethyl glycinate	108-110	54	4.80
8	H	$-CF_3$	H	Me	Me	0	0	D-Alanine methyl ester	Oil	43	4.58
9	H	$-CF_3$	H	H	Et	0	1	β-Alanine ethyl ester	Oil	42	5.13
10	H	$-CF_3$	H	$-CH_2$-COOMe	Me	0	0	L-Aspartate dimethyl ester	94-96	62	4.34
11	Cl	H	Cl	H	H	1	0	Ethyl glycinate	155-157	73	4.76
12	Cl	H	Cl	Me	Me	1	0	D-Alanine methyl ester	115-117	70	4.54
13	Cl	H	Cl	Me	Me	1	0	L-Alanine methyl ester	110-113	60	4.54
14	Cl	H	Cl	H	Et	1	1	β-Alanine ethyl ester	105-108	54	5.05
15	Cl	H	Cl	$-CH(Me)_2$	Me	1	0	D-Valine methyl ester	60-62	58	5.47
16	Cl	H	Cl	$-CH_2-CH(Me)_2$	Me	1	0	L-Leucine ethyl ester	72-74	65	6.03
17	Cl	H	Cl	$-CH_2$-COOMe	Me	1	0	L-Aspartate dimethyl ester	115-117	70	4.39

The IR spectra of the compounds **7-17** exhibited in each case, a band in the region ῡ1754-1627 cm^{-1} due to the carbonyl absorptions of amide and ester groups, whereas the absorption band of NH amide function appeared in the region ῡ 3421-3299 cm^{-1}, but it was obscured by strong and sharp NH secondary amine absorption at ῡ 3421-3292 cm^{-1}. In addition, compounds containing aspartic acid moiety, **10** and **17** exhibited an additional carbonyl absorption band within the same above mentioned region due to the additional ester moiety of aspartic acid. The ^{1}H NMR spectra of compounds **7-17** revealed the presence of two D_2O exchangeable NH signals in the regions δ 6.1-6.6 and δ 9.10-9.20 ppm due to amidic NH and Ar-NH-Ar, respectively in addition to the characteristic signals of diphenyl amine (aromatic moieties) around δ 7.07-7.45 ppm. Furthermore, the amino acid protons were distinguished in each molecule. Compounds **11-17** showed signal referring to the benzylic methylene group at δ 3.58 ppm, which appeared as singlet except for compounds **15** and **16** in which two doublets were observed with J =13.5-14 Hz indicating geminal coupling. ^{13}C NMR spectra of **11-17** exhibited the characteristic ^{13}C aromatic carbons signals of the diphenylamine moiety at δ 114.71-145.69 ppm, in addition to the distinguished signals of each amino acids esters carbons. Moreover, compounds **7-10** displayed a signal at δ 125.14 ppm (-**C**F3) while, **11-17** exhibited signal at δ 38.92 ppm (Ar**C**H2-). The structures of compounds **7-17** were further confirmed by two-dimensional (2D) NMR, ^{1}H,^{1}H-Homonuclear COSY NMR and ^{1}H-^{13}C-Heteronuclear COSY NMR. For example, their ^{1}H,^{1}H-

Homonuclear COSY NMR spectra showed the coupling between amide proton and the adjacent proton resulting in the splitting of NH signal. Moreover, X-ray single crystal analysis for compound **17** [17] gave an absolute confirmation for the proposed structure as shown in Figure 2.

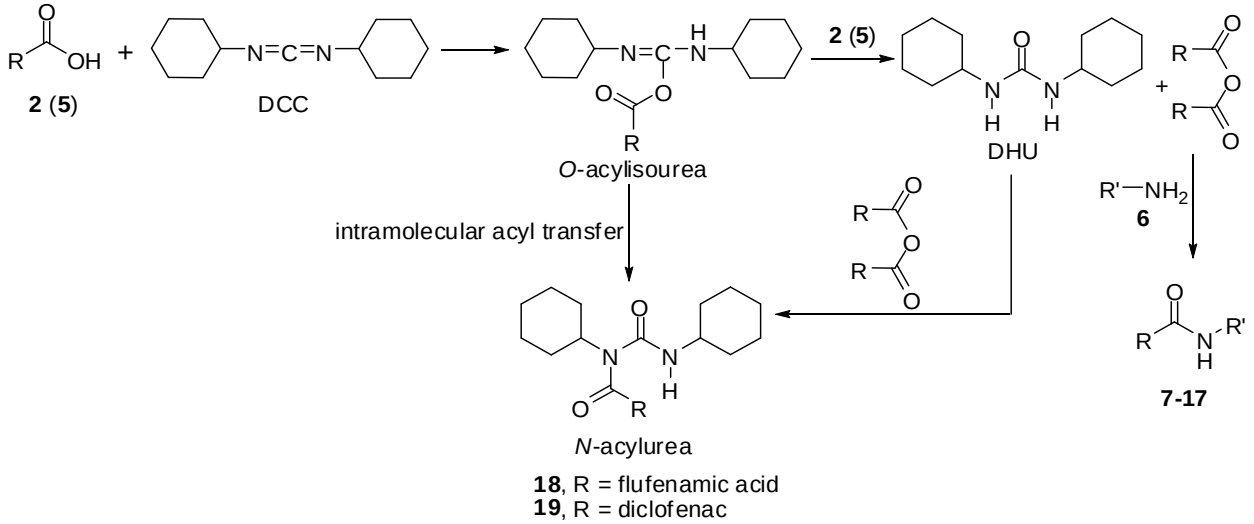
Figure 2: ORTEP diagram of compound 17



Dicyclohexylcarbodiimide (DCC) has been used to activate carboxyl groups of **2** and **5**. The initial step of the reaction is the addition of the carboxyl group to the carbodiimide function of DCC to provide the reactive acylating agent O-acylisourea and it subsequently reacts with the amino acid to form the desired amides and dicyclohexyl urea (DHU) (Figure 3).

However, in addition to the synthesized compounds **7-17** and DHU, other products were isolated in each case namely, the N-acylurea **18** and **19** of flufenamic acid and diclofenac, respectively, which were formed either; by the reaction between DHU and the activated symmetrical anhydride or from collapse of the O-acylisourea by intramolecular acyl transfer (Figure 3) .

Figure 3: Reaction pathways



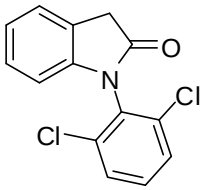
Compounds **18** and **19** provide, in each case molecular ion peaks corresponding to their molecular weights. On the other hand, the IR spectra revealed absorption bands at ῡ 1680 and 1650 cm^{-1} characteristic of the two amidic carbonyl groups.

^{1}H NMR spectra of **18** and **19** were consistent with the proposed structures, most interestingly the downfield appearance of the exchangeable NH proton at δ 6.33 ppm as doublet due to the coupling with CH of cyclohexyl ring,

which appeared at 3.57 ppm as multiplet. ^{13}C NMR data further confirmed the assignments that have already been made.

Interestingly, in case of diclofenac **5**, compound **20** (Figure 4) was isolated beside the target amides, DHU and N-acylurea **19**. Compound **20** was formed due to the intramolecular attack of the activated symmetrical anhydride to the its secondary amine NH to give the stable 1-(2,6-dichlorophenyl)indolin-2-one (**20**) as white powder with m.p = 115-117^{o}C. This attack didn't take place with flufenamic acid **2,** since this may lead to the formation of the strained four membered lactam ring.

Figure 4: Structure of compound 20



20

The IR spectrum of **20** showed the disappearance of the intense sharp absorption band at 3258 cm^{-1} assigned for (Ar-NH-Ar) in **5** and appearance of intense strained five-membered lactam carbonyl at ῡ 1731 cm^{-1}. ^{1}H NMR spectrum displayed signals at δ 3.89 ppm (-CH_2-) and at δ 6.38-7.76 ppm (7, ArH). ^{13}C NMR gave signals at δ 35.05 ppm (-CH_2-), 108-142.77 ppm (Ar-C) and 173.19 (C=O). Compound **20** exhibited a peak at m/z 278, which correspond to the molecular ion peak. The amount of the side produced **20** was greatly reduced and consequently the yield of the target compounds significantly increased by simultaneous addition of two equivalents of amines to the activated diclofenac **5**.

2.2. Biological investigations

2.2.1. In Vitro Antiproliferative Activity and Cell Cycle Disruption Effect

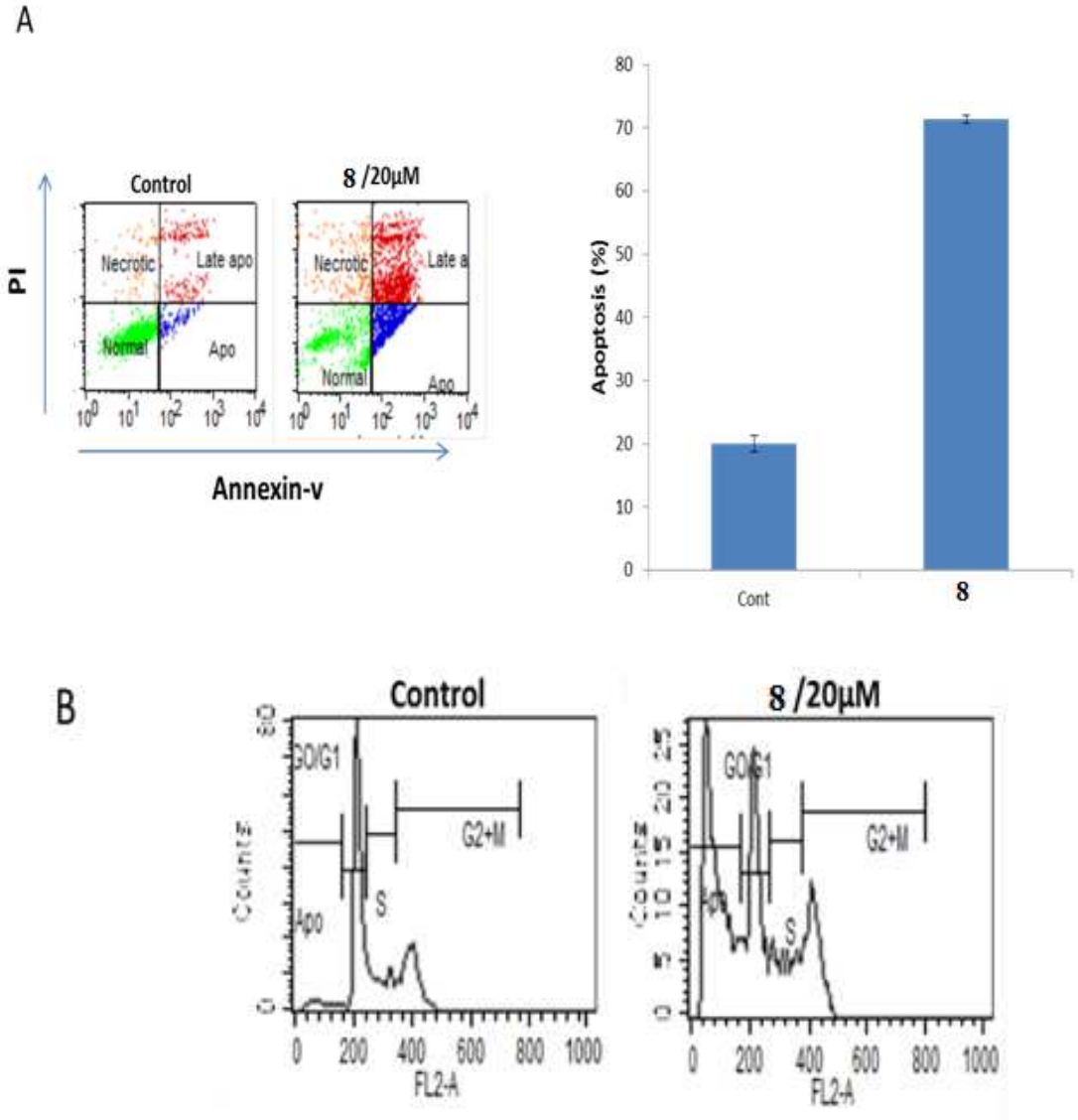
Antiproliferative activity in vitro was measured by the cell growth inhibition assay. The general in vitro cytotoxic evaluation of the synthesized compounds was conducted by using WST-1 reagent for determination of IC_{50} for each compound according to the protocol mentioned in the experimental section; results are given in Table 2. From the medicinal chemistry point of view, there is no constant pattern on the activity of these derivatives, however, **17** showed a good antiproliferative activity IC_{50} = 25.82 µM. On other hand, the other diclofenatc amide derivatives are inactive within 40 µM range concentration. Within flufenamic acid amide derivatives, **8** showed a potent antiproliferative activity with IC_{50} = 15.4 µM and the other derivatives were inactive within 40 µM range. Surprisingly, **8** disrupted the cell cycle and induced apoptosis with little effect on activation of cell death necrosis (Figure 5). Compounds **11**, **12**, **13** and **17** were more potent than the reference drug dichlorofluorescein diacetate (DCF) IC_{50}= 17.81 µM, while the IC_{50} for these compound ranges from 6.46-13.99 µM (Table 2). It was found that there is no evident relation between the tumor cell growth inhibitory activity of the tested compounds and their lipophilicity expressed as Clog P. Clearly, the lipophilicity has an influence on the activity, but it does not solely determine the antiproliferative activity of these compounds. These data were consistent with previously reported data that, carboxylic amide modification of the existing NSAIDs will impart potency and safety on these drugs as antiproliferative agents [14].

Table 2. Antiproliferative activity of the newly synthesized compounds against cancer cell lines

Compound	IC_{50} (µM)[a] HT-29	IC_{50} (µM)[a] A549
2	b	b
5	b	b
7	b	21.21
8	15.4±0.031	b
9	b	b
11	b	13.99
12	b	9.28
13	b	6.46
14	b	b
15	b	b
16	b	b
17	25.82±0.06	9.14
5-Fluorouracil	18±0.31	b
DCF	-	17.81

[a] IC_{50}: Concentration of the compound (µM) producing 50 % cell growth inhibition after 48h of compound exposure as determined by the WST-1 assay. Each experiment was run at least two times.[b] Inactive within 40µM concentration range

Figure 5: Cell cycle disruption and apoptotic effect of 8 on HT29-Cell line



A) HT-9 cells were harvested after treatment by **8** for 48h and incubated with annexin V-FITC and PI as described in "Materials and Methods". Ten thousand cells were analyzed per determination. Dot plots show Annexin V-FITC binding on the X axis and PI staining on the Y axis. Dots represent cells as follows: lower left quadrant, normal cells (FITC-/PI -); lower right quadrant, apoptotic cells (FITC+/PI-); upper left quadrant, necrotic cells (FITC-/PI+).

B) HT-29 cells were treated with or without compound in 20µM concentration and analyzed at 0h and 48h DNA flow cytometry. Histograms show the number of cells per channel (vertical axis) vs. DNA content (horizontal axis). The values indicate the percentage of cells in the relevant phases of the cell cycle. The analysis shown arresting of cells in G_0-phase by about 10 folds compared and S-phase by about 2 folds with untreated cells.

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