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Article

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Long Chain Alkyl Esters of Hydroxycinnamic Acids as Promising Anticancer Agents: Selective Induction of Apoptosis in Cancer Cells

Short title- Hydroxycinnamate alkyl esters induce apoptosis in cancer cells

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Abstract

Cancer is the major cause of morbidity and mortality worldwide. Hydroxycinnamic acids (HCAs) are naturally-occurring compounds and their alkyl esters may possess enhanced biological activities. We evaluated C4, C14, C16 and C18 alkyl esters of *p*-coumaric, ferulic, sinapic and caffeic acids (19 compounds) for their cytotoxic activity against four human cancer cells and also examined their effect on cell cycle alteration and apoptosis induction. The tetradecyl (**1c**) and hexadecyl (**1d**) esters of *p*-coumaric acid and tetradecyl ester of caffeic acid (**4c**), but not the parental HCAs, were selectively effective against MOLT-4 (human lymphoblastic leukemia) cells with IC_{50} values of 0.123 ± 0.012, 0.301 ± 0.069 and 1.0 ± 0.1 μM, respectively. Compounds **1c**, **1d,** and **4c** significantly increased apoptotic cells in sub-G1 phase and activated the caspase-3 enzyme in MOLT-4 cells. Compound **1c** was 15.4 and 23.6 times more potent than doxorubicin and cisplatin, respectively, against the drug resistant MES-SA-DX5 uterine sarcoma cells. These *p*-coumarate esters were several times less effective against NIH/3T3 fibroblast cells. Docking studies showed that **1c** may cause cytotoxicity by interaction with carbonic anhydrase IX. In conclusion, long chain alkyl esters of *p*-coumaric acid are promising scaffolds for selective apoptosis induction in cancer cells.

Keywords: Cancer cell, cytotoxic activity, hydroxycinnamic acids, apoptosis, caspase-3

57 **Abbreviations**

58

59 Hydroxycinnamic acids (HCAs)

60 *p*-Coumaric acid (*p*-CA)

61 Ferulic acid (FA)

62 Sinapic acid (SA)

63 Caffeic acid (CA)

64 Carbonic anhydrase IX (CA-IX)

65 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

66 Fetal bovine serum (FBS)

67 Phosphate buffered saline (PBS)

68 Dimethyl sulfoxide (DMSO)

69 Propidium Iodide (PI)

70 Poly(vinylidenedifluoride) (PVDF)

71 5-(1-(4-methylphenyl)-1H-1,2,3-triazol-4-yl)thiophene-2-sulfonamide (MPTTS)

72 Lamarckian Genetic Algorithm (LGA)

73 Root-Mean-Square Deviation (RMSD)

74 Hypoxia inducible factor-1α (HIF-1α)

75

1. Introduction

Cancer is the major cause of morbidity and mortality worldwide. Different types of cancer have caused 8.2 million deaths in 2012 and this number is escalating to 13.1 million in 2030[1]. Chemotherapy is the main treatment modality available to cancer patients. However, the resistance of cancer cells to chemotherapeutic agents and their side effects limits the use of these agents and therefore have constantly encouraged the discovery of novel antitumoral agents with better safety and efficacy profiles[2-3].

Hydroxycinnamic acids (HCA), or phenylpropanoids, including *p*-coumaric acid (**1a**), ferulic acid (**2a**), sinapic acid (**3a**) and caffeic acid (**4a**) (Table 1), are phenolic compounds naturally found in fruits and vegetables[4-6]. Hexadecyl (**1d**) and octadecyl (**1e**) esters of *p*-CA have been found in sweet potato[7]. Similarly, long chain alkyl esters of FA were identified in roots of *Ipomoea batatas*[8], while octadecyl caffeate (**4e**) and coumarate (**1e**) are both present in the root surface of sweet potato[9]. HCAs and their ester, amide, and glycoside derivatives have also shown various biological activities[5, 10-13]. In particular, anticancer effect of these natural phenolic compounds has been studied by several investigators[14-18].

One of the limiting factors for the pharmacological use of HCAs is their hydrophilicity, which hinders their efficiency to interact with cell membranes and subsequently lowers their efficacy[11-12]. Esterification can significantly increase the partition coefficient and lipophilicity and this may improve the compound's ability in crossing the membranes and lead to its higher pharmacological effectiveness. Previous reports from our and others' laboratories have also dealt with the effect of esterification on biological activities of HCAs. In this regard, different alkyl or aryl esters of HCAs have demonstrated enhanced *in vitro* antioxidant, neuroprotective and anti-

cancer potential compared to their parental compounds[12-13, 16-23]. The anticancer effects of FA and CA have been shown in several reports[17, 21, 24-25]; however, less attention has been focused on *p*-CA and its esters.

In our previous study, long chain alkyl esters of HCAs were synthesized and their antioxidant activity was assessed[13]. Following our ongoing program of the study of biological activities of HCAs [26] and in order to obtain new insights on their mechanism of action as anticancer agents, herein we evaluated the cytotoxic potential of long-chain alkyl esters of HCAs (**1b-1e**, **2b-2d**, **3b-3e** and **4b-4e**) against four different human cancer and one non-cancer cell lines. Their cytotoxic effects were investigated on cell cycle progression and apoptosis using flow cytometry and Western blotting, respectively. A molecular docking study was also performed for evaluating the anticancer potential of these HCAs as inhibitors of carbonic anhydrase IX (CA-IX).

2. Materials and Methods

2.1. Chemicals

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich (Saint Louis, MO, USA) and penicillin/streptomycin were purchased from Invitrogen (San Diego, CA, USA). Fetal bovine serum (FBS), phosphate buffered saline (PBS) and trypsin were purchased from Biosera (Ringmer, UK). Roswell Park Memorial Institute (RPMI) 1640 medium was from PAA (Austria). Doxorubicin was purchased from EBEWE Pharma (Unterach, Austria) and cisplatin from Mylan (Athens, Greece). Dimethyl sulfoxide (DMSO) and isopropanol were from Merck (Darmstadt, Germany).

The other chemical reagents and solvents utilized for synthesis were purchased from Sigma-Aldrich Química S.A. and Merck (Sintra, Portugal). Thin layer chromatography (TLC) was performed on silica gel 60 F254 precoated on aluminum plates acquired from Merck (Darmstadt, Germany) and spots were visualized under a UV lamp at 254 nm.

^{1}H, ^{13}C NMR, and DEPT135 data were recorded at room temperature on a Brüker AMX 300 spectrometer at 400 and 100 MHz, respectively. Mass spectra were obtained on a VG AutoSpec and microTOF (focus) mass spectrometer. A Biotage Initiator Microwave Synthesizer was used for synthesis. The purity of the final products (>97% purity) was verified by high-performance liquid chromatography (HPLC) equipped with a UV detector. Chromatograms were obtained in an HPLC/DAD system, a Jasco instrument (pumps model 880-PU and solvent mixing model 880-30, Tokyo, Japan), equipped with a commercially prepacked Nucleosil RP-18 analytical column (250 mm x 4.6 mm, 5 μm, Macherey-Nagel, Duren, Germany), and UV

detection (Jasco model 875-UV) at the maximum wavelength of 254 nm. The mobile phase consisted of a methanol/water or acetonitrile/water (gradient mode, room temperature) at a flow rate of 1 mL/min. The chromatographic data was processed in a Compaq computer, fitted with CSW 1.7 software (DataApex, Czech Republic).

2.2. Synthesis

2.2.1. Synthesis of butyl cinnamates (**1b**, **2b**, **3b,** and **4b**)

The butyl cinnamates were obtained by the following general procedure: The appropriate cinnamic acid (0.5 g), *n*-butyl alcohol (15 mmol) and 2 drops of concentrated sulphuric acid were mixed in a glass vial (5 mL) and sealed. The temperature of the reaction in the MW reactor was maintained at 20 ºC higher than the boiling point of butanol for 5 min. After the reaction the mixture was cooled to room temperature, and the crude product was extracted with ethyl acetate. The organic phase was washed with water, dried and the solvent evaporated. The residue was purified using silica gel flash chromatography and dichloromethane or ethyl acetate as eluent.

2.2.2. Synthesis of tetradecyl, hexadecyl, and octadecyl HCAs-

The long chain alkyl cinnamates were obtained by a two-step procedure: Step 1: Meldrum's acid and the appropriate alcohol in equimolar quantities were refluxed in dry toluene (5 mL) for 4 h. After the reaction mixture had cooled the crude product was extracted with diethyl ether. The ether phases were combined dried and then evaporated. These half esters of malonic acid were used in the next reaction without further purification. Step 2: The respective half ester of malonic acid and the appropriate benzaldehyde derivative in equimolar quantities

were added to cyclohexane (3-5 mL). Anhydrous pyridine (1 equiv.) and aniline (1.6 equiv.) were then, added to the mixture and it was refluxed for 6-10 h. After neutralization (pH-7), the crude products were extracted with diethyl ether. The organic phases were combined, dried and the solvent was evaporated. The residues were purified by flash chromatography using petroleum ether/ ethyl acetate mixtures. The short and long chain alkyl HCAs (**1b-1e**, **2b-2d**, **3b-3e** and **4b-4e**) structural assignments were in agreement with the previously reported data[12-13, 27-29].

(*E*)-Butyl-3-(4-hydroxyphenyl)propenoate **1b**; ^{1}H NMR (DMSO-d_6): δ 0.85 (t, 3H, J = 6.8 Hz, CH_3), 1.19 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 3.50 (t, 2H, J = 6.6 Hz, CH_2), 6.31 (d, 1H, J = 16.0 Hz, H-α), 6.65 (d, 2H, J = 8.7 Hz, H-3, 5), 7.50 (m, 3H, H-2, 6, β), 9.43 (s, 1H, OH); ^{13}C NMR (DMSO-d_6) δ 14.4 (CH_3), 18.9 (CH_2), 31.7 (CH_2), 64.2 (CH_2), 114.7 (C-α), 116.2 (C-3, 5), 125.6, 130.7 (C-2, 6), 145.0 (C-β), 160.3 (C-4), 167.1 (CO).

(*E*)-Tetradecyl-3-(4-hydroxyphenyl)propenoate **1c**[13, 28]; ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H, J = 6.6 Hz, H-14'), 1.26 (bs, 22H, H-3'-13'), 1.69 (m, 2H, H-2'), 4.19 (t, 2H, J = 6.6 Hz, H-1'), 5.81 (bs, 1H, OH), 6.30 (d, 1H, J = 15.9 Hz, H-α), 6.85 (d, 2H, J = 8.7 Hz, H-3, 5), 7.43 (d, 2H, J = 8.4 Hz, H-2, 6), 7.63 (d, 1H, J = 15.9 Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 14.1 (C-14'), 22.7 (C-13'), 26.0 (C-3'), 28.7 (C-2'), 29.27 (C-4'), 29.33 (C-11'), 29.6 (C-5'-10'), 31.9 (C-12'), 64.8 (C-1'), 115.5 (C-α), 115.9 (C-3, 5), 127.1 (C-1), 129.9 (C-2, 6), 144.5 (C-β), 157.9 (C-4), 167.9 (CO); HRESIMS: *m/z* 383.2554 $(M + Na)^+$; Calcd for $C_{23}H_{36}O_3Na^+$: 383.2557.

(*E*)-Hexadecyl-3-(4-hydroxyphenyl)propenoate **1d**[28]; ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H, J = 6.6 Hz, H-16'), 1.1-1.8 (m, 28H, H-2'-15'), 4.19 (t, 2H, J = 6.6 Hz, H-1'), 5.70 (bs, 1H, OH), 6.30 (d, 1H, J = 15.9 Hz, H-α), 6.85 (d, 2H, J = 8.6 Hz, H-3, 5), 7.42 (d, 2H, J = 8.5 Hz, H-2, 6), 7.62 (d, 1H, J = 15.9 Hz, H-β).

(*E*)-Octadecyl-3-(4-hydroxyphenyl)propenoate **1e**[28]; ^{1}H NMR ($CDC1_3$): 0.88 (t, 3H, *J* = 6.7 Hz, H-18'), 1.25-1.71 (m, 32H, H-2'-16'), 4.18 (t, 2H, *J* = 6.7 Hz, H-1'), 5.16 (s, 1H, OH), 6.30 (d, 1H, *J* = 15.9 Hz, H-α), 6.84 (d, 2H, *J* = 8.7 Hz, H-3, 5), 7.44 (d, 2H, *J* = 8.6 Hz, H-2, 6), 7.62 (d, 1H, *J* = 15.9 Hz, H-β).

(*E*)-Butyl-3-(4-hydroxy-3-methoxyphenyl)propenoate **2b**[12]; ^{1}H NMR ($CDCl_3$): δ 0.97 (t, 3H, *J* = 7.4 Hz, CH_3), 1.39-1.49 (m, 2H, CH_2), 1.65-1.72 (m, 2H, CH_2), 3.92 (s, 3H, OCH_3), 4.20 (t, 2H, *J* = 6.7 Hz, CH_2), 5.97 (s, 1H, OH), 6.29 (d, 1H, *J* = 15.9 Hz, H-α), 6.92 (d, 1H, *J* = 8.1 Hz, H-5), 7.03 (d, 1H, *J* = 1.8 Hz, H-2), 7.07 (dd, 1H, *J* = 8.1, 1.8 Hz, H-6), 7.61 (d, 1H, *J* = 15.9 Hz, H-β); ^{13}C NMR (DMSO-d_6): δ 14.7 (CH_3), 20.1 (CH_2), 31.7 (CH_2), 56.8 (OCH_3), 65.2 (CH_2), 110.2 (C-5), 115.6 (C-2), 116.5 (C-α), 123.9 (C-6), 127.9 (C-1), 145.6 (C-β), 147.7 (C-4), 158.1 (C-3), 168.3 (CO).

(*E*)-Tetradecyl-3-(4-hydroxy-3-methoxyphenyl)propenoate **2c**[13]; ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 3H, *J* = 6.6 Hz, H-14'), 1.25 (bs, 22H, H-3'-13'), 1.68 (m, 2H, H-2'), 3.93 (s, 3H, OCH_3), 4.18 (t, 2H, *J* = 6.7 Hz, H-1'), 5.87 (bs, 1H, OH), 6.29 (d, 1H, *J* = 15.9 Hz, H-α), 6.91 (d, 1H, *J* = 8.1 Hz, H-5), 7.03 (s, 1H, H-2), 7.07 (dd, 1H, *J* = 8.1, 1.8 Hz, H-6), 7.60 (d, 1H, *J* = 15.9 Hz, H-β).

(*E*)-Octadecyl-3-(4-hydroxy-3-methoxyphenyl)propenoate **2e**[13]; ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 3H, *J* = 6.6 Hz, H-18'), 1.25 (bs, 30H, H-3'-17'), 1.68 (m, 2H, H-2'), 3.93 (s, 3H, OCH_3), 4.19 (t, 2H, *J* = 6.8 Hz, H-1'), 5.86 (bs, 1H, OH), 6.30 (d, 1H, *J* = 15.9 Hz, H-α), 6.90 (d, 1H, *J* = 8.1 Hz, H-5), 7.04 (s, 1H, H-2), 7.07 (dd, 1H, *J* = 8.1, 1.8 Hz, H-6), 7.60 (d, 1H, *J* = 15.9 Hz, H-β).

(*E*)-Butyl-3-(3,5-dimethoxy-4-hydroxyphenyl)propenoate **3b**[27]; ^{1}HNMR (DMSO-d_6): δ 0.91 (t, 3H, *J* = 7.3 Hz, CH_3), 1.33-1.42 (m, 2H, CH_2), 1.56-1.64 (m, 2H, CH_2), 3.80 (s, 6H, 2 ×

203 OCH_3), 4.12 (t, 2H, *J* = 6.4 Hz, CH_2), 6.53 (d, 1H, *J* = 15.9 Hz, H-α), 7.03 (s, 2H, H-2, 6), 7.55
204 (d, 1H, *J* = 15.9 Hz, H-β), 8.94 (s, 1H, OH); ^{13}C NMR (DMSO-d_6): δ 13.6 (CH_3), 18.8 (CH_2),
205 30.4 (CH_2), 56.1 (2 × OCH_3), 63.4 (CH_2), 106.2 (C-2, 6), 115.0 (C-α), 124.4 (C-1), 138.3 (C-4),
206 145.3 (C-β), 148.0 (C-3, 5), 166.7 (CO); EI-MS, 280 (88,$M^{\bullet+}$).

207 (*E*)-Tetradecyl-3-(3,5-dimethoxy-4-hydroxyphenyl)propenoate **3c**[13]; ^{1}H NMR ($CDCl_3$): δ
208 0.87 (t, 3H, *J* = 6.6 Hz, H-14'), 1.25 (bs, 22H, H-3'-13'), 1.69 (m, 2H, H-2'), 3.91 (s, 6H, 2 ×
209 OCH_3), 4.18 (t, 2H, *J* = 6.6 Hz, H-1'), 5.81 (s, 1H, OH), 6.30 (d, 1H, *J* = 15.9 Hz, H-α), 6.77 (s,
210 2H, H-2, 6), 7.58 (d, 1H, *J* = 15.9 Hz, H-β); ^{13}C NMR ($CDCl_3$): δ 14.1 (C-14'), 22.7 (C-13'), 26.0
211 (C-3'), 28.8 (C-2'), 29.29 (C-4'), 29.33 (C-11'), 29.6 (C-5'-10'), 31.9 (C-12'), 56.3 (2 × OCH_3),
212 64.6 (C-1'), 105.1 (C-2, 6), 116.1 (C-α), 126.0 (C-1), 137.1 (C-4), 144.8 (C-β), 147.2 (C-3, 5),
213 167.21 (CO); HRESIMS: *m/z* 443.2762 $(M + Na)^+$; Calcd for $C_{25}H_{40}O_5Na^+$: 443.2768.

214 (*E*)-Hexadecyl-3-(3,5-dimethoxy-4-hydroxyphenyl)propenoate **3d**[13]; ^{1}H NMR ($CDCl_3$): δ
215 0.87 (t, 3H, *J* = 6.6 Hz, H-16'), 1.25 (bs, 26H, H-3'-15'), 1.69 (m, 2H, H-2'), 3.91 (s, 6H, OCH_3 ×
216 2), 4.18 (t, 2H, *J* = 6.6 Hz, H-1'), 5.80 (s, 1H, OH), 6.30 (d, 1H, *J* = 15.9 Hz, H-α), 6.77 (s, 2H,
217 H-2, 6), 7.58 (d, 1H, *J* = 15.9 Hz, H-β); ^{13}C NMR ($CDCl_3$): δ 14.1 (C-16'), 22.7 (C-15'), 26.0 (C-
218 3'), 28.8 (C-2'), 29.3 (C-4'), 29.33 (C-13'), 29.7 (C-5'-12'), 31.9 (C-14'), 56.3 (OCH_3 × 2), 64.6
219 (C-1'), 105.1 (C-2, 6), 116.1 (C-α), 126.0 (C-1), 137.1 (C-4), 144.78 (C-β), 147.2 (C-3, 5), 167.2
220 (CO). HRESIMS: *m/z* 471.3078 $(M + Na)^+$; Calcd for $C_{27}H_{44}O_5Na^+$: 471.3081.

221 (*E*)-Octadecyl-3-(3,5-dimethoxy-4-hydroxyphenyl)propenoate **3e**[13, 28]; ^{1}H NMR ($CDCl_3$): δ
222 0.87 (t, 3H, *J* = 6.6 Hz, H-18'), 1.25 (bs, 30H, H-3'-17'), 1.70 (m, 2H, H-2'), 3.92 (s, 6H, 2 ×
223 OCH_3), 4.19 (t, 2H, *J* = 6.6 Hz, H-1'), 5.78 (s, 1H, OH), 6.30 (d, 1H, *J* = 15.9 Hz, H-α), 6.77 (s,
224 2H, H-2, 6), 7.59 (d, 1H, *J* = 15.9 Hz, H-β); ^{13}C NMR ($CDCl_3$): δ 14.1 (C-18'), 22.7 (C-17'), 26.0
225 (C-3'), 28.8 (C-2'), 29.3 (C-4'), 29.33 (C-15'), 29.7 (C-5'-14'), 31.9 (C-16'), 56.3 (2 × OCH_3), 64.6

(C-1'), 105.1 (C-2, 6), 116.1 (C-α), 126.0 (C-1), 137.1 (C-4), 144.8 (C-β), 147.2 (C-3, 5), 167.24 (CO); HRESIMS: *m/z* 499.3398 $(M + Na)^+$; Calcd for $C_{29}H_{48}O_5Na^+$: 499.3394.

(*E*)-Butyl-3-(3,4-dihydroxyphenyl)propenoate **4b**[12]; ^{1}H NMR (DMSO-d_6): δ 0.90 (t, 3H, J = 7.5 Hz, CH_3), 1.32-1.42 (m, 2H, CH_2), 1.56-1.65 (m, 2H, CH_2), 4.11 (t, 2H, J = 6.6 Hz, CH_2), 6.27 (d, 1H, J = 15.9 Hz, H-α), 6.76 (d, 1H, J = 8.1 Hz, H-5), 7.00 (dd, 1H, J = 8.1, 2.0 Hz, H-6), 7.05 (d, 1H, J = 2.0 Hz, H-2), 7.46 (1H, d, J = 15.9 Hz, H-β), 9.60 (2H, s, 3, 4-OH); ^{13}C NMR (DMSO-d_6): δ 23.3 (CH_3), 28.4 (CH_2), 40.0 (CH_2), 73.2 (CH_2), 123.8 (C-5), 124.3 (C-2), 125.5 (C-α), 131.2 (C-6), 135.2 (C-1), 154.7 (C-β), 155.3 (C-OH), 158.1 (C-OH), 176.5 (CO).

(*E*)-Tetradecyl-3-(3,4-dihydroxyphenyl)propenoate **4c**[13, 29]; ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H, J = 6.6 Hz, H-14'), 1.26 (bs, 22H, H-3'-13'), 1.67 (m, 2H, H-2'), 4.18 (t, 2H, J = 6.7 Hz, H-1'), 5.65 (s, 1H, OH), 5.66 (s, 1H, OH), 6.27 (d, 1H, J = 15.9 Hz, H-α), 6.87 (d, 1H, J = 8.1 Hz, H-5), 7.02 (dd, 1H, J = 8.1, 1.9 Hz, H-6), 7.09 (d, 1H, J = 1.8 Hz, H-2), 7.57 (d, 1H, J = 15.9 Hz, H-β); ^{13}C NMR ($CDCl_3$): δ 14.0 (C-14'), 22.6 (C-13'), 25.9 (C-3'), 28.4 (C-2'), 29.2 (C-4', 11'), 29.6 (C-5'-10'), 31.8 (C-12'), 64.7 (C-1'), 114.3 (C-α), 115.4 (C-5), 115.9 (C-2), 122.3 (C-6), 127.7 (C-1), 143.6 (C-3), 144.4 (C-β), 146.0 (C-4), 167.5 (CO); HRESIMS: *m/z* 399.2508 $(M + Na)^+$; Calcd for $C_{23}H_{36}O_4Na^+$: 399.2506.

(*E*)-Hexadecyl-3-(3,4-dihydroxyphenyl)propenoate **4d**[13, 29]; ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 3H, J = 6.6 Hz, H-16'), 1.25 (bs, 26H, H-3'-15'), 1.67 (m, 2H, H-2'), 4.18 (t, 2H, J = 6.7 Hz, H-1'), 5.69 (bs, 2H, 2 × OH), 6.27 (d, 1H, J = 15.9 Hz, H-α), 6.87 (d, 1H, J = 8.1 Hz, H-5), 7.02 (dd, 1H, J = 8.1, 1.8 Hz, H-6), 7.09 (d, 1H, J = 1.8 Hz, H-2), 7.57 (d, 1H, J = 15.9 Hz, H-β); ^{13}C NMR (DMSO-d_6): δ 13.4 (C-16'), 21.6 (C-15'), 24.9 (C-3'), 27.7 (C-2'), 28.1 (C-4', 13'), 28.5 (C-5'-12'), 30.8 (C-14'), 63.2 (C-1'), 113.5 (C-α), 114.3 (C-5), 115.2 (C-2), 120.8 (C-6), 125.0 (C-1),

144.5 (C-3), 145.1 (C-β), 147.9 (C-4), 166.12 (CO); HRESIMS: *m/z* 427.2815 $(M + Na)^+$; Calcd for $C_{25}H_{40}O_4Na^+$: 427.2819.

(*E*)-Octadecyl-3-(3,4-dihydroxyphenyl)propenoate **4e**[13, 29]; ^{1}H NMR (DMSO-d_6): δ 0.84 (t, 3H, *J* = 6.6 Hz, H-18'), 1.22 (bs, 30H, H-3'-17'), 1.61 (m, 2H, H-2'), 4.09 (t, 2H, *J* = 6.5 Hz, H-1'), 6.25 (d, 1H, *J* = 15.9 Hz, H-α), 6.75 (d, 1H, *J* = 8.1 Hz, H-5), 7.0 (dd, 1H, *J* = 8.1, 1.8 Hz, H-6), 7.04 (d, 1H, *J* = 1.8 Hz, H-2), 7.45 (d, 1H, *J* = 15.9 Hz, H-β), 9.17 (s, 1H, OH), 9.61 (s, 1H, OH); ^{13}C NMR (DMSO-d_6): δ 13.4 (C-18'), 21.6 (C-17'), 24.9 (C-3'), 27.8 (C-2'), 28.2 (C-4', 15'), 28.5 (C-5'-14'), 30.8 (C-16'), 63.2 (C-1'), 113.5 (C-α), 114.2 (C-5), 115.2 (C-2), 120.8 (C-6), 125.0 (C-1), 144.5 (C-β), 145.1 (C-3), 147.9 (C-4), 166.1 (CO); HRESIMS: *m/z* 455.3135 $(M + Na)^+$; Calcd for $C_{27}H_{44}O_4Na^+$: 455.3132.

2.3.*Cell lines*

HL-60 (human promyelocytic leukemia), MCF-7 (human breast adenocarcinoma), MOLT-4 (human acute lymphoblastic leukemia) and NIH/3T3 (mouse embryonic fibroblast) cells were obtained from the National Cell Bank of Iran at Pasteur Institute, Tehran, Iran. MES-SA/DX5 (human sarcoma cells multi-resistant) cells were purchased from Sigma-Aldrich. All cells were grown in monolayer cultures and maintained at 37 °C in humidified air containing 5% CO_2 except for HL-60 and MOLT-4 cells that were grown in suspension. Cells were maintained in RPMI 1640 containing 10% FBS, and 100 units/mL of penicillin-G and 100 μg/mL of streptomycin. Only for HL-60 cells, RPMI 1640 containing FBS at the concentration of 20% was used.

2.4. Cytotoxicity assay

271

272 The cytotoxic activities of the compounds were evaluated by MTT reduction assay [30-31].
273 One hundred μL of cell suspension having a density of 50,000 cells/mL were seeded into each
274 well of a 96-well microplate and incubated overnight at 37 °C. Three to four concentrations of
275 compounds, ranging from 50 nM to 100 μM, determined by the potency of the compound, were
276 added to the wells in triplicate and the plates were incubated for another 72 h at 37 °C. All
277 compounds were dissolved in DMSO, except for compounds **2e**, **3e** and **4e**, which were
278 solubilized in isopropanol. The final concentrations of DMSO or isopropanol in the wells did not
279 exceed 0.25%. This concentration of the solvents did not affect the viability of the cell lines
280 tested in this study. At the end of the incubation, the growth medium was replaced with 0.5
281 mg/mL of MTT dissolved in fresh RPMI 1640 without phenol red. After an additional 4 h of
282 incubation at 37 °C, the formazan crystals were dissolved in 200 μL DMSO and the optical
283 absorbance was measured at 570 nm (applying a background correction at 655 nm) using a Bio-
284 Rad microplate reader (Model 680). The cell viability expressed as percent inhibition for each
285 concentration of the compound was calculated with reference to the control and the IC_{50} values
286 were calculated with the CurveExpert software version 1.34 for Windows. Each experiment was
287 repeated 3-4 times.

288

289 *2.5. Cell cycle analysis*

290 The analysis of cells in different phases of the cell cycle and the sub-G1 phase were
291 monitored using propidium iodide (PI)-based assessment of cell cycle by flow cytometry.
292 MOLT-4 cells were seeded in 12-well plates (1×10^6 cells /well) and treated with different
293 concentrations of **1c** (30, 100 and 300 nM), **4c** (300, 1000 and 3000 nM) and **1d** (100, 300 and

1000 nM) for 24 and 48 h. At the end of the incubation, the cells in each well were collected and washed with PBS. Then, they were fixed with 70% ethanol overnight at -20 °C. After 24 h, the fixed cells were washed with PBS and subsequently stained with DNA staining solution (PI 20 μg/mL and RNase 200 μg/mL) at room temperature for 30 min in the dark. Twenty thousand cells of each sample were analyzed using a FACS Calibur flow cytometer (BD Biosciences, USA) and the percentage of the cells in sub-G1, G0/G1, S, and G2/M phases were calculated using CellQuest (BD, USA) software.

2.6. Preparation of cell lysates for immunoblotting

MOLT-4 cells were seeded at a density of 250,000 cells/mL in 12-well plates and were incubated at 37 °C for 24 h. The alkyl ester derivatives of HCAs were introduced into the cell cultures and they were further incubated for 24 h. The cells were harvested and lysed for 30 min in ice-cold RIPA lysis buffer (Tris-HCl 250 mM, NaCl 750 mM, NP-40 5%, EDTA 5 mM, sodium deoxycholate 2.5 %, SDS 0.5%) with frequent vortex mixing. PMSF and a protease inhibitor cocktail (Roche) were added to the extraction buffer in order to inhibit the protease enzymes. The lysates were centrifuged at 15,000 g for 20 min at 4 °C. Supernatants were isolated in fresh tubes and stored at -80 °C before use. Protein contents of the cell extracts were measured by a BCA protein assay kit (Quanti-Pro ®BCA, Sigma–Aldrich, St. Louis, USA) using bovine serum albumin as the protein standard.

2.7. Immunoblotting of caspase-3

317 The activation of the caspase-3 enzyme was detected by immunoblotting. Twenty μg of
318 total proteins were loaded on 12% SDS-polyacrylamide gels and electrophoresis was performed
319 at a constant voltage. The proteins were then electrotransferred onto poly(vinylidene difluoride)
320 (PVDF) membranes and non-specific binding sites were blocked with 5% fat-free milk dissolved
321 in Tris-buffered saline containing Tween 20 (TBS-T, 150 mM NaCl and 50 mM Tris-HCl pH
322 7.4, 0.1% Tween 20) at room temperature for 40 min. Proteins were then detected by specific
323 primary antibodies, rabbit monoclonal anti-caspase-3 (dilution 1:400, clone 8G10, Cell
324 Signaling, USA) and anti-actin (dilution 1:2000, Sigma-Aldrich, USA) overnight at 4 ^{o}C.
325 Membranes were washed three times in TBST buffer and then incubated with secondary
326 antibody (goat anti-rabbit horseradish peroxidase conjugated IgG, dilution 1:1000, Santa Cruz
327 Biotechnology) for 1 h at room temperature. After 3 further washes in TBST buffer,
328 immunoreactive bands were visualized using enhanced chemiluminescence detection substrate
329 (Thermo Fisher Scientific, Waltham, MA). Images were acquired with an image acquiring
330 system (G: BOX Chemi, Syngene, Cambridge, U.K.) with GeneSnap software. The density of
331 the protein bands was quantified by GeneTools software (Syngene), followed by normalization
332 to the β-actin as a loading control. All experiments were replicated at least three times.
333
334 *2.8. Target prediction for anticancer activity of HCAs*
335
336 The method that we applied for exploring the plausible molecular targets responsible for
337 the anti-cancer potential of HCAs, consisted of the following steps[32]:
338 1. Extraction of molecular targets of interest for HCAs from Drug Bank, ChemMapper [33],
339 DINIES[34], SuperPred[35] and BindingDB[36],

2. Gathering targets based on highest structural and physiochemical similarities of their ligands with HCA derivatives.

3. Selecting molecular targets involved in the mechanism of anti-cancer action based on CancerResource[33, 37].

4. Text mining with the purpose of investigation of the exact molecular target (among selected targets in steps 2 and 3) involved in the anticancer activity of the test compounds.

2.9. Molecular docking

A molecular docking study of alkyl HCAs was conducted in order to further explore the mechanisms of cytotoxicity as inhibitors of CA-IX and evaluation of their structural requirement for the binding interaction with this potential target. The X-ray crystal data of three-dimensional structure of human CA-IX in complex with 5-(1-(4-methylphenyl)-1H-1,2,3-triazol-4-yl)thiophene-2-sulfonamide (MPTTS, PDB ID: 5FL6) was acquired from the Protein Data Bank (http://www.rcsb.org). The crystal structure of the protein was further refined by removing the co-crystallized ligand and water molecules and the protein was converted to pdbqt format using Autodock Tools (1.5.4). The active site was derived from the X-ray crystal structure data. The 3D coordinates of studied ligands were generated by using Chem3D Ultra 8 and energetically minimized by the conjugate gradient algorithm (gradient convergence value of 0.1 $kcalmol^{-1}$ was used) using the HyperChem software. The Gastiger charges (empirical atomic partial charges) were calculated by HyperChem software and the torsional degrees of freedom were assigned on the PDB files. The pdbqt files were generated for each ligand separately using ADT 1.5.4. The grid maps of the active site for enzyme were calculated by Auto-Grid with three dimensions of

363 60 × 60 × 60 (x, y, z), grid spacing of 0.375 Å and the cubic grids were centered on the binding
364 site of MPTTS as the cognate ligand.
365 Docking computations were performed using the Lamarckian genetic algorithm (LGA).
366 For this purpose, maximum numbers of evaluations were set to 2,500,000, the numbers of GA
367 runs were 50 and the maximum numbers of generations were set as 27,000 and all other options
368 were set as default. Resulting conformers differing by less than 1.0 Å in positional root-mean-
369 square deviation (RMSD) were clustered together and represented by the result with the top-
370 ranked free energy of binding interaction.
371
372 *2.10. Statistical analysis*
373
374 The data were expressed as the mean ± S.D. or S.E.M. and were analyzed by one-way
375 ANOVA using the SPSS software version 14.0 for Windows with LSD post hoc test, when
376 applicable.
377

3. Results

3.1. Cytotoxicity of alkyl hydroxycinnamates against cancer cells

Cytotoxic activities of hydroxycinnamic acids (HCAs) including **1a**, **2a**, **3a**, **4a** and their medium and long chain alkyl ester derivatives (butyl "**b**", tetradecyl "**c**", hexadecyl "**d**" and octadecyl "**e**" esters, Table 1) were evaluated against 4 cancer cell lines (HL-60, MCF-7, MOLT-4 and MES-SA/DX5) and on the non-cancer NIH/3T3 cells using MTT reduction assay. The cell viability assay results were compared with cisplatin and doxorubicin, which were used as standard anticancer agents (Table 2).

3.2. Effect of alkyl hydroxycinnamates on cancer cell cycle

In order to explore the plausible mechanism of cytotoxicity of the most potent HCA esters, the cell-cycle distribution of MOLT-4 cells incubated with **1c**, **1d** and **4c** were evaluated using propidium iodide (PI)-based assessment of cell cycle by flow cytometry after 24 and 48 h. Figures 1 and 2 illustrate the distribution of the cells in the G0/G1, S and G2/M phases of cell cycle. The results indicated that the number of apoptotic cells in sub-G1 phase was significantly increased after treatment with different concentrations of test compounds after 24 and 48 h. The cell cycle profile of MOLT-4 cells treated with increasing concentrations of **1c** at 30, 100 and 300 nM after 24 h indicated that the percentage of cells in sub-G1 phase showed a dose-dependent increase compared to control cells. Similarly, exposure of cells to **1c** for 48 h resulted in a concentration-dependent increase in the percentage of apoptotic cells. Similar patterns of

dose- and time-dependent responses were observed in a sub-G1 fraction of cells treated with **1d** and **4c**.

3.3. Effect of alkyl hydroxycinnamates on the activation of caspase-3

Three of the most effective alkyl hydroxycinnamates were incubated with MOLT-4 cancer cells for 24 h and the total protein was extracted and analyzed by immunoblotting for determination of caspase-3 activation, which was verified by the cleavage of caspase-3 and the appearance of a smaller 17 KD fragment. As shown in Figure 3, exposure of MOLT-4 cells to **1c** (0.5 μm), **4c** (2.5 μm) and **1d** (2.5 μm) resulted in the cleavage of caspase-3 and the appearance of the cleaved caspase-3 peptide (17 KD), accompanied by reduction of pro-caspase-3 (35 KD) protein.

3.4. Carbonic anhydrase IX as the potential molecular target for alkyl hydroxycinnamates

All linear alkyl ester derivatives of HCAs were modeled with Hyperchem 7. Then targets were collected from target prediction databases, through a process schematically shown in Figure 4. We used a text-mining method and examined experimental applications to cancer studies based on the existing literature. As the final finding, carbonic anhydrase isoforms were identified as putative targets for HCA derivatives.

3.5. Molecular modeling study: interaction of HCAs in complex with carbonic anhydrase IX

The crystal structure of CA-IX (PDB code 5FL6) in complex with MPTTS was used for molecular docking study. For validation of the docking procedure, RMSD (root mean square) of experimental conformer was calculated by re-docking of MPTTS in CA-IX's active sites. The top-ranked conformer of MPTTS into the binding site of CA-IX superimposed well over the X-ray coordinates of experimental one with the RMSD value of 1.03Å (Figure 5).

Docking results and comparative binding interactions of **1a** and **1c**, into CA-IX complex, are depicted in Figure 6B and 6A, respectively. Based on the docking results, the corresponding binding free energies of **1a** and **1c** into CA-IX were -5.35 and -5.80 Kcal/mol, respectively. Compound **1a** showed 3 hydrogen binding interactions with Asn66, His68 and Thr200 of CA-IX. Esterification of the acidic moiety of compound **1c** resulted in favorable hydrogen bonding interactions with Asn66, His68, Gln71 and Thr200 of CA-IX catalytic site, which was observed in all CA-IX inhibitors. Moreover, **1c** was also positioned in a close interaction (less than 3Å) with Leu199 and Thr201, which are conserved residues in the catalytic site of all carbonic anhydrases.

Comparing the binding interaction of **1c** with its methoxylated counterparts **2c** (ΔG= -5.21 Kcal/mol) and **3c** (ΔG= -4.77 Kcal/mol), it could be concluded that introduction of methoxy substitution into the phenyl ring might distort the binding orientation of ester pendant into CA-IX catalytic site and hinder the hydrogen bonding interaction with key residues such as Asn66, His68, and Gln71. Compound **2c** takes part in hydrogen bonding interactions with Thr200 and Gln92, while **3c** only interacted with Thr200. Dislocation of tetradecyl ester side chain was

446 demonstrated in the case of **2c** and **3c** (Figure 6C and 6D). Moreover, addition of hydroxyl
447 moieties into the phenyl ring of HCAs, also had negative effects on the binding orientation of the
448 compound in the CA-IX active site; **4c** (containing two hydroxyl groups at R2 and R3 position),
449 is devoid of any H-bond interactions with Asn66, His68, and Gln71, and is only involved in H-
450 bond interaction with Thr200, Thr201, and Gln92 (Figure 6E).
451

452 ## Discussion

453

454 Cytotoxic activities of hydroxycinnamic acids (HCAs) and their medium and long chain
455 alkyl ester derivatives were evaluated against 4 human and one non-cancer cell line using MTT
456 reduction assay. The effects of the most potent compounds were examined on cell cycle and
457 induction of apoptosis.

458 The MTT assay results showed that among different tested HCA derivatives, tetradecyl
459 (**1c**) and hexadecyl (**1d**) esters of *p*-CA were the most potent compounds against MOLT-4 cells
460 with IC_{50} values of 0.123 and 0.317 μM, respectively. Interestingly, these derivatives showed
461 IC_{50} values (0.317 and 0.414 μM, respectively) against multidrug resistant cell line MES-
462 SA/DX5 that were much lower than those of doxorubicin (1.9 μM) and cisplatin (2.9 μM),
463 standard chemotherapeutic agents (Table 2). Doxorubicin and cisplatin are commonly used
464 chemotherapeutic agents for treatment of several solid tumors and leukemias and have been
465 utilized by us and other authors as standard cytotoxic agents. All *p*-CA derivatives were inactive
466 against HL-60 and MCF-7 cells, which shows that these compounds may specifically affect
467 certain cancer cell lines and not others. On the basis of the obtained cytotoxic data, the following
468 structure-activity relationships could be deduced for the synthesized compounds of this study:

469 *Influence of aromatic substitution pattern on cytotoxic activity*

470 In general, *p*-CA ester derivatives (**1b-e**) showed higher cytotoxic effects against MOLT-
471 4 and MES-SA/DX5 cells, followed by caffeate (**4b-e**) and ferulate esters (**2b-e**), while sinapate
472 esters (**3b-e**) showed the lowest anticancer effects against these cells.

473 The number of hydroxyl functions and the nature of *m*-substituents on the aromatic ring
474 seems to influence the cytotoxic activity of the test compounds. In this case, the presence of a
475 single 4-OH function was the most favorable substitution pattern (as in *p*-CA derivatives),
476 followed by the presence of a 3,4-dihydroxy (catechol) moiety (as in CA derivatives).

477 The results obtained in HL-60, MCF-7 cells do not allow a straightforward evaluation of
478 the influence of the aromatic substitution pattern due to lower activity levels. However, CA (**4a**)
479 and its ester derivatives (**4b-e**), bearing a catechol moiety, were the only group of compounds
480 that showed a moderate activity against HL-60 cells.

481 *Effect of carboxylic acid esterification on cytotoxicity*

482 Esterification of the carboxylic acid function generally improved the cytotoxic activity of
483 all HCA derivatives against MOLT-4 and MES-SA/DX5 cells. For instance, *p*-CA (**1a**) was
484 inactive in these cellular models (IC_{50} > 100 μM), while the ester derivatives **1b-e** showed a
485 significant improvement in cytotoxic effect against these cell lines (Table 2).

486 *Length of alkyl chain and physicochemical properties*

487 With the exception of butyl caffeate (**4b**) in HL-60 cells, tetradecyl esters were the most
488 active derivatives in all cancer cellular models. The existence of an optimal chain length points
489 towards specific structural and physicochemical requirements that allow effective transport
490 and/or diffusion across the cell membrane and interaction with intracellular targets. Among
491 them, lipophilicity (as determined by the partition coefficient LogP, Table **1**), stands out as an

492 important physicochemical descriptor of bioactivity. As shown in Table **1**, the naturally-
493 occurring carboxylic acids (**1a**, **2a**, **3a** and **4a**) are the most hydrophilic compounds in this series
494 (1.36 < Log P < 1.87), which may hinder their cellular uptake and intracellular accumulation,
495 resulting in the poor cytotoxic activity observed. Accordingly, esterification of the carboxylic
496 acid moiety improved the derivatives' lipophilicity, as could be observed in increased LogP
497 values. However, chain lengths longer than the optimal size may increase the interaction with the
498 phospholipids in the membrane and decrease the solubility in the intracellular compartments,
499 which may justify the observed decrease in cytotoxicity in the current cellular models. This
500 observation was in accordance with previous reports regarding the antioxidant activity and
501 membrane affinity of FA ester derivatives[38-39]. Similar observation of a biphasic correlation
502 between lipophilicity and anticancer activity of caffeate esters has also been reported [29]. In the
503 present case, the tetradecyl ester moiety may provide optimal membrane anchoring, orienting the
504 HCA moiety towards its intracellular targets and thus providing maximum activity.

505 Other authors have screened the effect of different alkyl esters ranging from C3 to C22 of
506 CA and FA against different cancer cell lines. They identified C12 and C16 caffeate esters, and
507 also C8 and C12 ferulate esters as the optimum alkyl chain length against MCF-7, HCT-116
508 (colon adenocarcinoma), CCI-H460 (lung adenocarcinoma), SF-26 (CNS tumor) and AGS
509 (gastric adenocarcinoma) cells with an IC_{50} range of 2.5 to 18.7 μg/ml. The C14 ester was not
510 included in this study and the authors did not find any preferential effect against a certain type of
511 cancer [21].

512 It has been previously shown that hexyl caffeate and hexyl ferulate exhibit cytotoxicity
513 against certain types of cancer including MCF-7 cells[18]. Uwai and colleagues have shown that
514 C11-C14 caffeate esters have the highest cytotoxicity against Raw264.7 macrophage cells, with

C11 ester being the most potent in inhibition of NO in these cells[29]. The biphasic correlation between ClogP, the index of lipophilicity, and cytotoxicity indicated that the cytotoxicity of caffeate esters was directly correlated with lipophilicity, having an optimum range[29].

The effect of these compounds against cancer cells was also compared with non-cancer cells. Derivatives **1c** and **1d**, with remarkable submicromolar potency against MOLT-4 and MES-SA/DX5 cells, were almost devoid of cytotoxicity against non-malignant NIH/3T3 cells. Moreover, *meta* substitution with hydroxyl group could affect the selectivity of compounds against cancer cells; Although the presence of a 3,4-dihydroxy (catechol) group in the aromatic ring broadened the cytotoxic activity spectrum to HL-60 cells (**4b**-**e**, Table **2**), it also induced toxic effects against non-cancer cells, which raises selectivity issues.

We also examined the effect of the most active compounds on cell cycle; At 300 nM the percentage of sub-G1 cells exposed to **1c** for 24 and 48 h (27.0 and 54.7%, respectively) were higher than the percentages related to compounds **4c** (4.9 and 6.5%, respectively) and **1d** (17.7 and 34.7%, respectively). Therefore based on these findings, induction of apoptosis could be considered as a conceivable mechanism of anticancer activity of these HCA esters.

On the other hand, caspase-3 assessment results were in accordance with cell cycle analysis that evidenced the induction of apoptosis by compounds **1c**, **1d**, and **4c**. Caspase-3 is an executioner caspase and its activation was one of the signs of apoptosis induction in cells[25, 40-42].

It has been shown that FA was capable of apoptosis induction in prostate cancer cells PC-3 and LNCaP[24]. However, very high doses (300-500 μM) were tested in this study, which cannot be compared to our findings on FA and its esters. In a recent study, methyl-caffeate showed induction of apoptosis in MCF-7 cells, involving the caspase-3 and poly (ADP-ribose) polymerase-1 (PARP1) activation[25].

538 Various studies have shown that inhibition of matrix metalloproteinase (MMP) and
539 vascular endothelial growth factor (VEGF)[15, 43-44]; inhibition of NF-*k*B, Bcl-2; activation of Bax,
540 Bak and JNK[19, 45]; cell cycle arrest by inhibition of cyclin D1, E, B1 and induction of p16, p21,
541 p17[46-48] are the common mechanisms involved in the anti-proliferative and apoptosis inducing
542 effects of HCA derivatives.

543 In our target prediction study, carbonic anhydrase isoforms were identified as putative
544 targets for HCA derivatives, which was also corroborated by previous literature reports[49].
545 Carbonic anhydrases are abundant zinc metalloenzymes that catalyze the reversible
546 interconversion of CO_2 and HCO_3^- [50]. Several different carbonic anhydrase isoforms have been
547 identified, among which CA-IX, a membrane-associated glycoprotein, may have an important
548 role in cancer [51] and was overexpressed as a result of hypoxia in many cancer cells [52-53] through
549 the activation of hypoxia inducible factor-1α (HIF-1α) [54]. CA-IX was considered to be a good
550 candidate for cancer targeted therapy, as its expression was very limited in normal tissues, while
551 it was highly expressed in various cancers[55]. Different studies have shown that the cytotoxic
552 activity of phenolic acid esters including CA, FA, *p*-CA as well as other phenolic compounds is
553 possibly due to the inhibition of carbonic anhydrase[49, 56-57].

554 From the findings of the docking study, it might be concluded that esterification improves
555 the binding pose of HCAs into the CA-IX binding site, however, this improvement does not
556 seem to be sufficient to explain the dramatic effect of esterification on the cytotoxicity as it was
557 observed in the case of **1c** compared to **1a** against MOLT-4 and MES-SA/DX5 cells. The
558 superior cytotoxic potential of tetradecyl and other ester derivatives over the parental compounds
559 could also be largely attributed to the improved physicochemical properties by esterification,
560 increased lipophilicity and cell membrane permeability of HCAs. Alternatively, it could be

assumed that these compounds have additional mechanisms of action, through which, the considerable increase of activity caused by esterification could be explained.

Regarding the pharmacokinetic properties of HCAs, it has been shown that free *p*-CA has high bioavailability after oral intake but data for its conjugated forms is limited[58]. It was further observed that *p*-CA as a metabolite from its ester conjugates was detected at lower concentrations in plasma indicating its different metabolic pathway compared to the free *p*-CA[58]. The free forms of HCA are easily absorbed in the upper gastrointestinal tract[59]. For example, detection of 24% of *p*-CA 24h after its administration has shown less metabolism and faster uptake of this free HCA compared to cinnamic acid[60]. An extensive review of the pharmacokinetics and biological activities of HCA discusses the high rate of absorption of free *p*-CA and FA compared to CA from concluded from findings of various studies[6].

In an *in vivo* study, *p*-CA and gallic acid were orally administered to rats and their blood concentrations and pharmacokinetic profiles were measured[61]. It was found that *p*-CA was quickly absorbed from the gastrointestinal tract and 10 minutes after oral administration, the portal vein serum concentration of intact p-CA was 165.7 µM. Interestingly, this study reported that the bioavailability of *p*-CA was 70 times higher than gallic acid[61]. On the other hand, another study in Beagle dogs has shown that maximum plasma concentration of FA is 0.977 µM[62].

Since, long chain alkyl esters of HCAs are way more lipophilic compared to the parental compounds, it would be reasonable to presume that these compounds are absorbed into the lymphatic circulation, due to their lipophilic nature[63]. On the other hand, since the lymphatic system transports lipophilic compounds, it can be suggested that incorporation of the HCAs into

583 nanostructured lipid carriers may further increase their accumulation in the lymphatic system [64-
584 65].

585 In conclusion, the findings of this study demonstrate that long chain alkyl ester
586 derivatives of *p*-CA (compounds **1c** and **1d**) show remarkable submicromolar potency against
587 certain cancer cells and are selective towards these cells over non-malignant cells. Therefore,
588 they represent promising scaffolds as anticancer agents that selectively induce apoptosis in
589 cancer cells.

590

591 **Author contributions**

592

593 J.C.J.M.D.S.M: Performed experiments, wrote the paper; N.E.: Designed the study, wrote
594 the paper; S.P.K.: Performed experiments; M.K: Performed experiments; Z.K.: Performed
595 experiments; H.H.M.: Performed experiments; R.M.: Designed the study, wrote the paper; N.E.:
596 Designed the study, wrote the paper; M.N.: Performed experiments; J.A.S.C.: Performed
597 experiments; T.S.: Performed experiments, wrote the paper; L.S.: Designed the study, wrote the
598 paper; F.B. and O.F.: Designed the study, supervised the project, wrote the paper.

599

600

601 **Conflict of interest statement**

602

603 The authors hereby declare that they have no conflict of interest.

604

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Figure captions

Figure 1. Effect of HCA ester derivatives on the cell cycle distribution of MOLT-4 cells.

MOLT-4 cells were treated with various concentrations of compounds **1c** (**A**), **4c** (**B**) and **1d** (**C**) and for 24 and 48 h. At the end of the incubation time, the cells were collected and fixed with 70% ethanol overnight at -20 °C. After 24 h, the fixed cells were stained with DNA staining solution (PI 20 μg/mL and RNase 200 μg/mL) at room temperature for 30 min in the dark. Twenty thousand cells of each sample were analyzed using a FACS Calibur flow cytometer.

Figure 2. Alterations of percentages of MOLT-4 cells in each cell cycle phase after treatment with HCA esters.

The percentages after treatment with compounds **1c** (**A**), **4c** (**B**) and **1d** (**C**) are shown.

Figure 3. Effect of HCA esters on activation of caspase-3 in MOLT-4 cells.

MOLT-4 cells were incubated with compounds **1c** (0.5 μM), **4c** (2.5 μM) and **1d** (2.5 μM) for 24 h. Cells were lysed and cell lysate was analyzed by immunoblotting. The ratios of pro-caspase and cleaved caspase bands to actin were calculated and compared to control. Data are expressed as mean ± S.E.M of at least 3 independent experiments. *: Pro-caspase/actin ratio of the test compound was significantly different from the same ratio in the control ($P < 0.005$); #: Cleaved caspase/actin ratio of the test compound was significantly different from the same ratio in the control ($P < 0.05$).

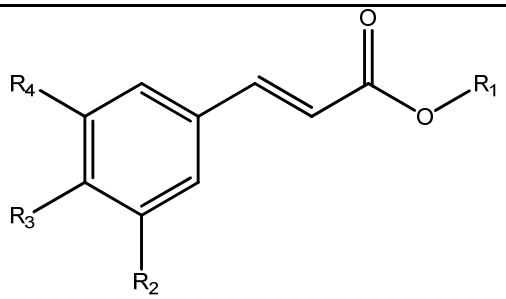
Figure 4. The schematic workflow of our methodological approach for finding plausible molecular targets involved in the anti-cancer activity of HCAs.

Drug targets (DTs) were obtained by collecting information from different target prediction databases in the different phases of the drug discovery. Data extracted from these databases were used to predict the potential targets.

Figure 5. Comparison of docked and crystallographic conformers into carbonic anhydrase IX active-site.

839 The protein key residues are shown in the line style, the crystallized conformer of the
840 ligand (5-(1-(4-methylphenyl)-1H-1,2,3-triazol-4-yl)thiophene-2-sulfonamide) is colored by its
841 atom type, while the docked conformer is demonstrated in yellow (D). For sake of clarity, only
842 catalytic residues are shown in this figure (see text for detail). The graphic image was produced
843 using the UCSF Chimera program (University of California, San Francisco).
844
845 **Figure 6. Binding interactions of HCA derivatives with carbonic anhydrase IX.**
846 Binding interactions of compounds **1c** (A), **1a** (B), **2c** (C), **3c** (D) and **4c** (E) with
847 conserved catalytic residues of CA-IX are depicted. Residues are shown as lines, while ligands
848 are shown in colored sticks. The carbon backbone of **1c** is depicted in green in all panels, while
849 the other compounds are superimposed on **1c** in panels B-E.
850

Table 1. Chemical structures and physicochemical properties of HCA ester derivatives

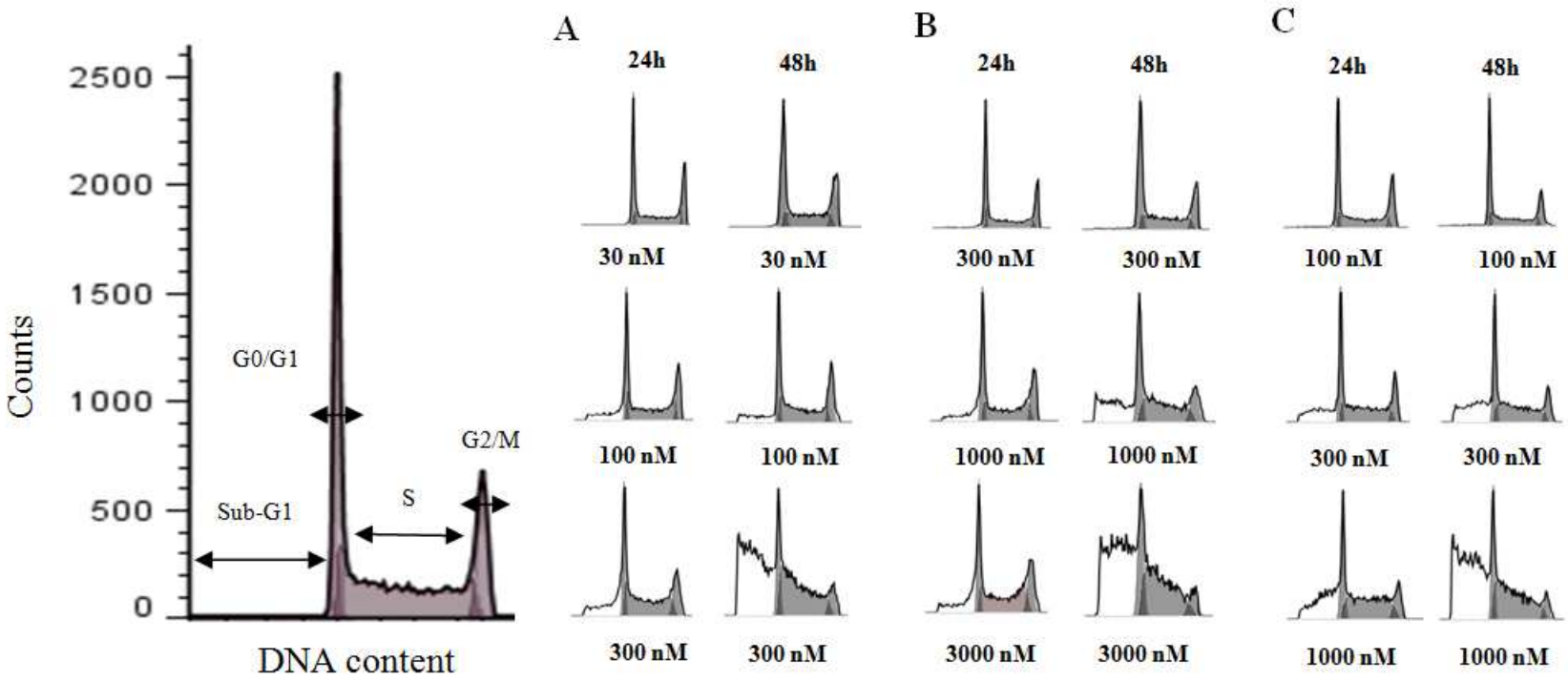


Compound	R_1	R_2	R_3	R_4	MW	LogP
			p-Coumaric acids			
1a	H	H	OH	H	164.16	1.87
1b	C_4H_9	H	OH	H	220.11	3.05
1c	$C_{14}H_{29}$	H	OH	H	360.537	7.07
1d	$C_{16}H_{33}$	H	OH	H	388.591	7.86
1e	$C_{18}H_{37}$	H	OH	H	416.64	8.15
			Ferulic acids			
2a	H	OCH_3	OH	H	194.19	1.62
2b	C_4H_9	OCH_3	OH	H	250.29	2.86
2c	$C_{14}H_{29}$	OCH_3	OH	H	390.56	6.82
2e	$C_{18}H_{37}$	OCH_3	OH	H	446.67	8.42
			Sinapic acids			
3a	H	OCH_3	OH	OCH_3	224.21	1.36
3b	C_4H_9	OCH_3	OH	OCH_3	280.32	2.60
3c	$C_{14}H_{29}$	OCH_3	OH	OCH_3	420.59	6.57
3d	$C_{16}H_{33}$	OCH_3	OH	OCH_3	448.32	7.80
3e	$C_{18}H_{37}$	OCH_3	OH	OCH_3	476.70	9.59
			Caffeic acids			
4a	H	OH	OH	H	180.16	1.58
4b	C_4H_9	OH	OH	H	236.27	2.82
4c	$C_{14}H_{29}$	OH	OH	H	376.54	6.79
4d	$C_{16}H_{33}$	OH	OH	H	404.59	7.58
4e	$C_{18}H_{37}$	OH	OH	H	432.64	8.37

Table 2. Cytotoxic activities of HCA esters against different human cancer cells and a non-cancer cell line (NIH/3T3) assessed by MTT assay.

Compound	IC_{50} (μM)				
	HL-60	**MCF-7**	**MOLT-4**	**MES-SA/DX5**	**NIH/3T3**
1a	>100	>100	>100	>100	>100
1b	>100	>100	50.9 ± 13.9	63.8 ± 7.2	>100
1c	>100	>100	0.123 ± 0.012	0.317 ± 0.088	32.4 ± 5.2
1d	>100	>100	0.301 ± 0.069	0.414 ± 0.129	>100
1e	>100	>100	3.8 ± 0.9	1.6 ± 0.2	>100
2a	>100	>100	>100	>100	>100
2b	>100	>100	>100	66.2 ± 8.5	>100
2c	54.4 ± 5.1	38.8 ± 6.9	0.860 ± 0.193	5.4 ± 0.7	33.8 ± 2.4
2e	>100	>100	7.6 ± 2.7	3.9 ± 0.4	>100
3a	>100	>100	>100	>100	>100
3b	>100	>100	>100	50.2 ± 4.9	>100
3c	>100	35.6 ± 4.1	33.2 ± 1.4	22.1 ± 1.7	47.1 ± 9.4
3d	>100	>100	72.9 ± 19.5	22.2 ± 1.8	>100
3e	>100	>100	20.7 ± 9.6	19.1 ± 4.1	>100
4a	26.1 ± 10.1	>100	>100	>100	>100
4b	9.4 ± 3.1	>100	9.9 ± 1.4	53.1 ± 9.6	21.4 ± 6.5
4c	15.0 ± 0.3	31.4 ± 9.1	1.0 ± 0.1	2.8 ± 0.3	4.3 ± 1.1
4d	29.0 ± 9.2	>100	2.0 ± 0.5	5.1 ± 1.8	7.9 ± 1.4
4e	36.7 ± 2.1	>100	5.5 ± 1.9	4.3 ± 1.4	9.2 ± 1.1
Doxorubicin	0.014 ± 0.004	0.043 ± 0.003	0.017 ± 0.003	1.9 ± 0.7	0.163 ± 0.095
Cisplatin	3.1 ± 0.2	2.0 ± 0.2	3.1 ± 0.1	2.9 ± 0.5	6.9 ± 3.5

Values are expressed as mean ± S.D of 3-5 experiments.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1500
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Counts
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G2/M
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DNA content
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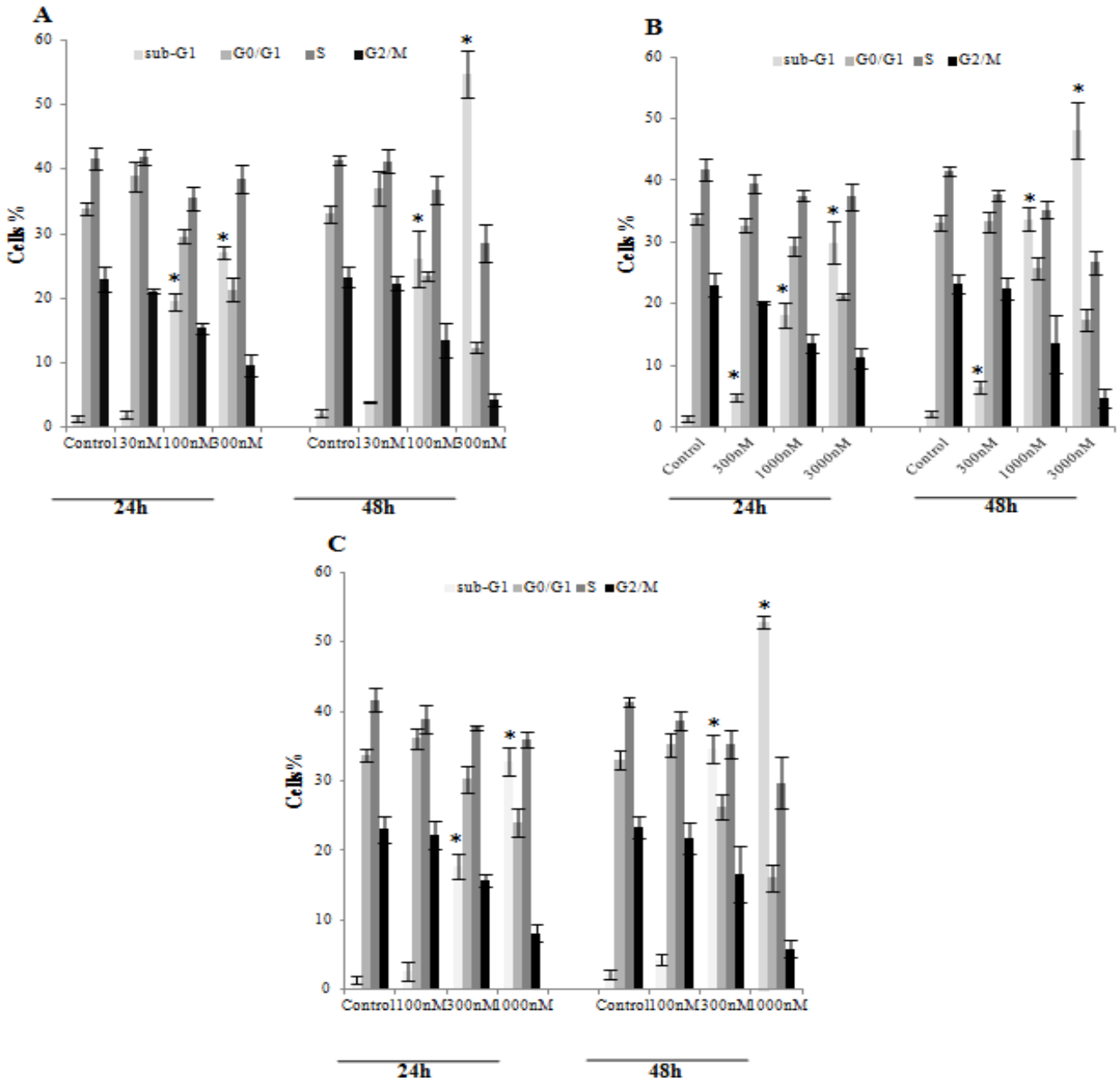
24h
48h
30 nM
100 nM
300 nM
1000 nM
3000 nM

Figure 1.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G0/G1
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G2/M
Cells %
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48h
B
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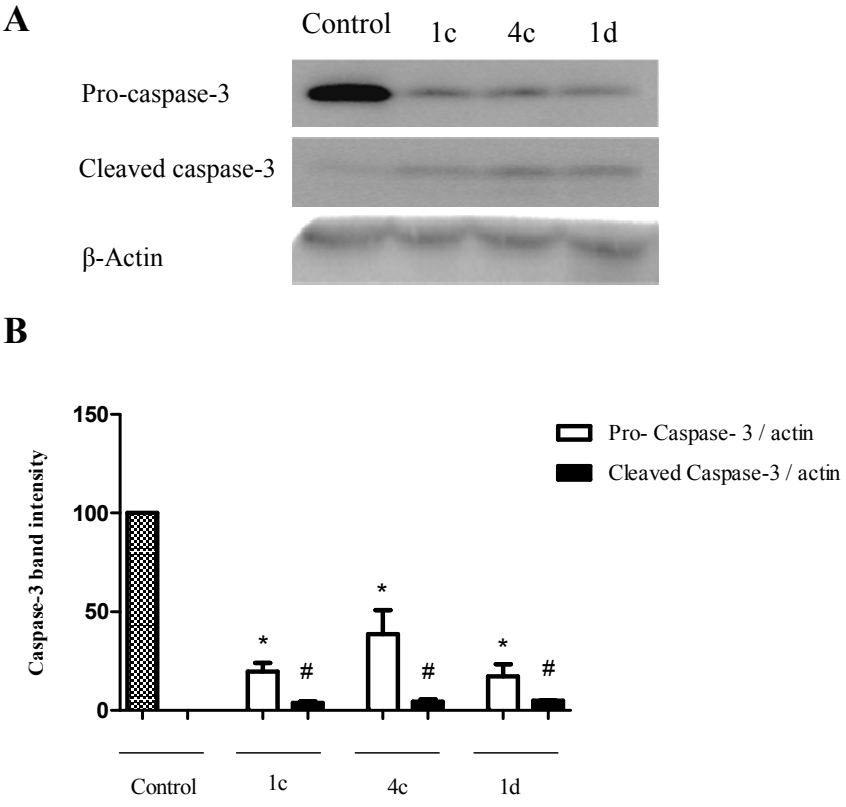
300nM
1000nM
3000nM
C
Control
100nM
300nM
1000nM

Figure 2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1d
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B
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Cleaved Caspase-3 / actin
*
#
Control
1c
4c
1d

Figure 3

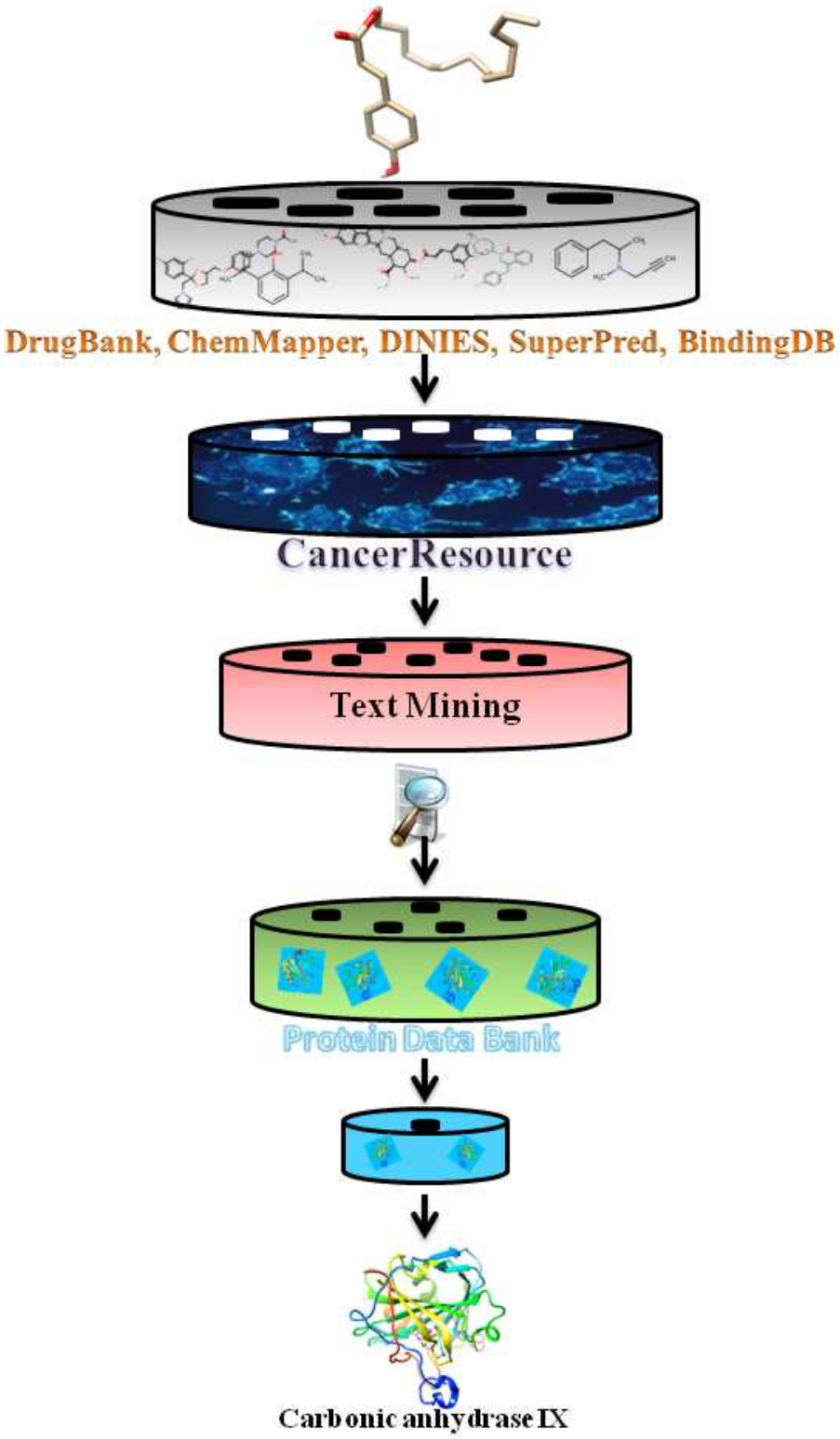

DrugBank, ChemMapper, DINIES, SuperPred, BindingDB
CancerResource
Text Mining
Protein Data Bank
Carbonic anhydrase IX

Figure 4

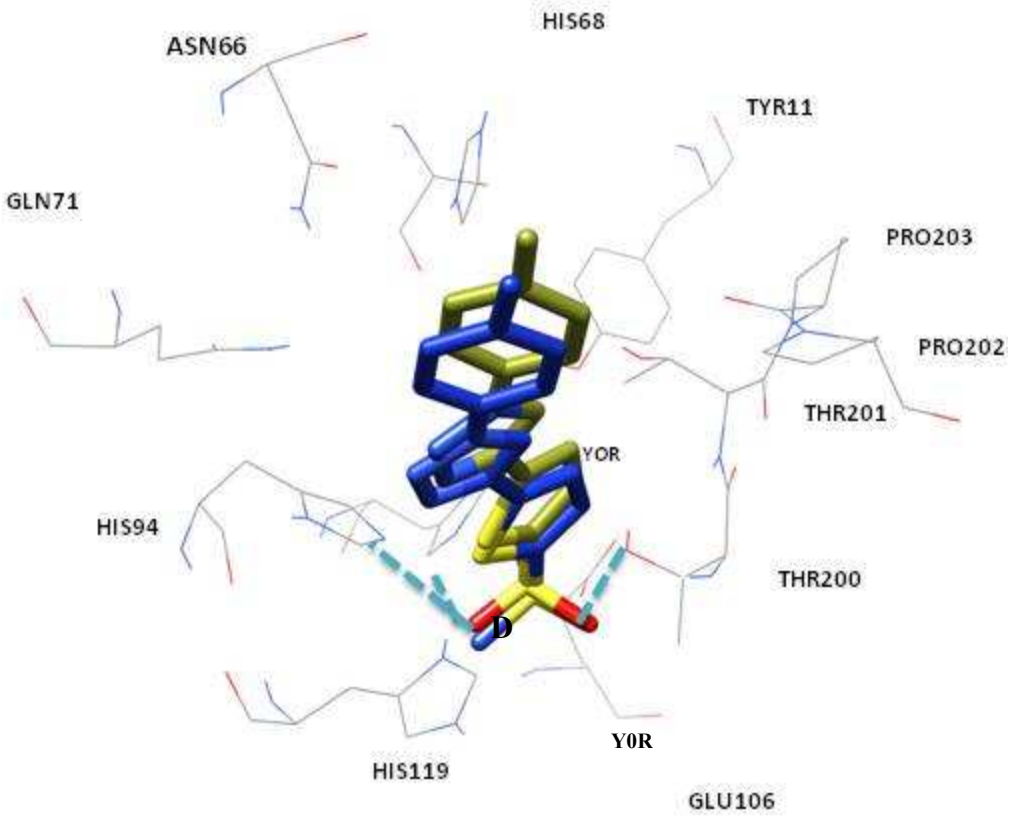

HIS68
ASN66
TYR11
GLN71
PRO203
PRO202
THR201
YOR
HIS94
THR200
D
Y0R
HIS119
GLU106

Figure 5

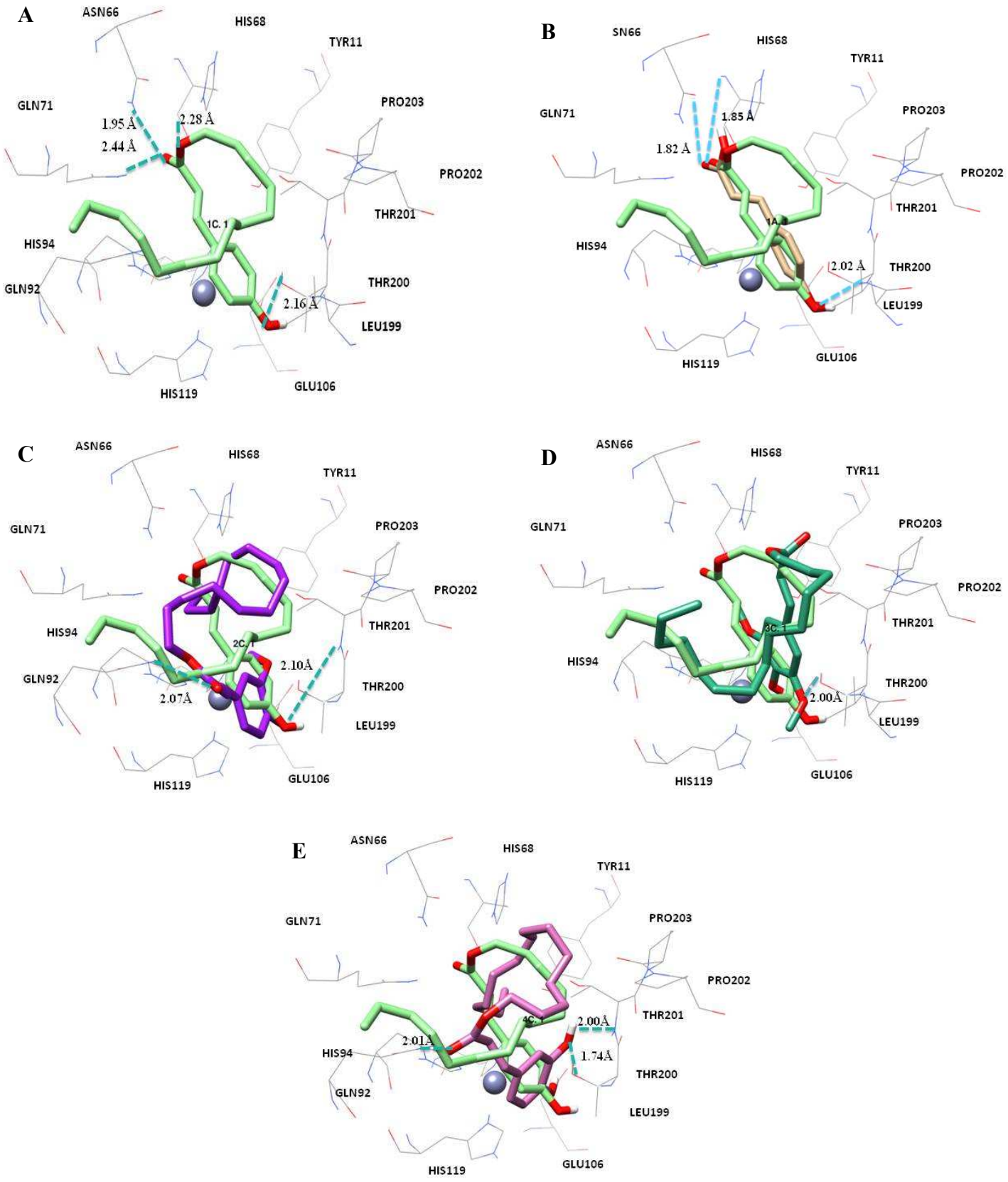
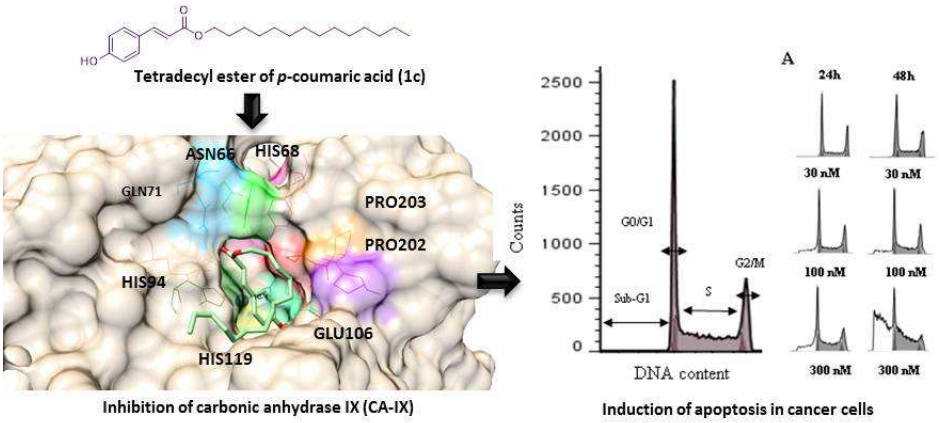

A
ASN66
HIS68
TYR11
GLN71
PRO203
1.95 Å
2.28 Å
2.44 Å
PRO202
THR201
HIS94
THR200
GLN92
2.16 Å
LEU199
HIS119
GLU106
B
SN66
HIS68
TYR11
GLN71
PRO203
1.85 Å
1.82 Å
PRO202
THR201
HIS94
2.02 Å
THR200
LEU199
HIS119
GLU106
C
ASN66
HIS68
TYR11
GLN71
PRO203
PRO202
THR201
HIS94
2.10Å
GLN92
2.07Å
THR200
LEU199
HIS119
GLU106
D
ASN66
HIS68
TYR11
GLN71
PRO203
PRO202
THR201
HIS94
THR200
2.00Å
LEU199
HIS119
GLU106
E
ASN66
HIS68
TYR11
GLN71
PRO203
PRO202
THR201
2.00Å
2.01Å
HIS94
1.74Å
THR200
GLN92
LEU199
HIS119
GLU106

Figure 6

TOC Graphic-



On screening of 19 derivatives of hydroxycinnamic acids, long chain alkyl ester derivatives of *p*-coumaric acid induced apoptosis in certain cancer cells (IC_{50}: 0.123-1.6 μM), conceivably by carbonic anhydrase IX inhibition.