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Facile synthesis, characterization and cytotoxicity study of new 3-(indol-2-yl) bicyclotetrazatridecahexaens

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Abstract: A new series of thiosemicarbazone-based indole derivatives **12-15** has been prepared by condensation reaction of indole-2-carboxamide derivatives **8-11** with thiosemicarbazide. The latter compounds underwent intracyclization in the presence of chloroacetic acid and sodium acetate to afford a set of new 3-(indol-2-yl)bicyclotetrazatridecahexaens **16-19**. These newly synthesized compounds have been characterized by means of FT-IR, ^1H NMR, ^{13}C NMR, HRMS, and by elemental analyses. Cytotoxic activities of the prepared compounds along with LY294002 were evaluated *in vitro* against normal human skin fibroblast, human colon carcinoma (HCT116), and leukemia (K562) cell lines; results revealed that the series inhibits only HCT116 cell line. In addition, results showed that compound **18** exerts moderate potency in HCT116 with an IC_{50} value of 54 μM and significantly induces apoptosis.

Key words: Synthesis, Thiosemicarbazones, Tetrazatridecahexaens, Cytotoxic activity, HCT116.

Introduction

The indole nucleus represents a significant heterocyclic core structure and is present in a wide range of bioactive compounds. A number of indole-containing drugs were prepared (Fig. 1) and tested as anticancer agents (1, 2).¹

Fig. 1. Chemical structures of indole-based molecules employed as anticancer agents.

On the other hand, the amide moiety in drugs plays an important role in drug-receptor complex formation; the presence of carbonyl groups permits carboxamides to serve as hydrogen-bond acceptors, whereas in primary and secondary amides they act as hydrogen-bond donors.²

Schiff bases exhibit a wide spectrum of biological activities including anticancer, anti-inflammatory, antibacterial, and antifungal.³ Regarding anticancer activity, the thiosemicarbazones of vanillin, benzophenones (3), and acetophenones significantly reduce average tumor weight and inhibit tumor cell growth in mice bearing Ehrlich ascites carcinoma (EAC) xenografts⁴ (Fig. 2). In this context, thiosemicarbazone derivatives prepared from acetylpyrazines with thiosemicarbazide (4) were tested for antitumor activity against SK-N-MC neuroepithelioma cells.⁵ Furthermore, many thiosemicarbazone derivatives were developed as highly efficient tyrosinase inhibitors (5-7).⁶⁻⁹

Fig. 2. Chemical structures of selected thiosemicarbazone-based molecules.

Our endeavor in targeting human colon carcinoma (HCT116) and myeloid leukemia (K562) cell lines has been recently culminated with new scaffolds: *N*-phenyl-4-hydroxy-2-quinolone-3-carboxamides,¹⁰ substituted-benzofuran-2-carboxamides,² indole-2-carboxamides,¹¹ and derivatives of benzoin.¹² As part of our ongoing research in the synthesis of heterocyclic compounds with pharmacological interest, the present work describes the synthesis and characterization of a new series of compounds having an indole-2-carboxamide core structure tailored with a motif bearing thiosemicarbazone. In addition, the biological activity of these newly prepared compounds were evaluated *in vitro* against human colon carcinoma (HCT116) and myeloid leukemia (K562) cell lines. To the best of our knowledge, these compounds have not been previously prepared.

Experimental method

All starting materials and reagents were purchased from Sigma-Aldrich Co. and used without further purification. Experiments were performed in purified solvents and the progress of reactions was monitored by TLC using pre-coated silica gel glass plates (E. Merck Kiesegel 60 F₂₅₄, layer thickness 0.25 mm). Melting points were determined using a Stuart Scientific electrothermal melting point apparatus and were uncorrected. Nuclear

magnetic resonance (NMR) ^1H NMR, ^{13}C NMR and DEPT spectra were acquired by a Bruker 500 MHz-Avance-III instrument operating at 500.13 (^1H) and 125.03 MHz (^{13}C) relative to TMS as internal standard, J values for ^1H - ^1H coupling constants are given in Hertz (Hz). Infrared (IR) spectra were recorded on an Avatar Thermo Nicolet Impact 400 FT-IR spectrophotometer using the Smart Omni-Transmission software; all samples were prepared as potassium bromide (Acros, Belgium) discs. High-resolution mass spectra (HRMS) were obtained on electrospray ion trap (ESI) technique by collision-induced dissociation on a Bruker APEX-IV (7 Tesla) instrument. External calibration was performed using an arginine cluster at a mass range of m/z 175-871, and the samples were dissolved in chloroform. Elemental analyses were obtained using Elemental analyzer model EA3000 A, Italy.

General procedure for synthesis of 12-15

A mixture of thiosemicarbazide (6.8 mmole), 0.5 mL of concentrated hydrochloric acid and indole-2-carboxamide derivatives **8-11**¹¹ (2.3 mmol) in 20 mL ethanol was refluxed for 24 h, then the resulted solution was cooled to room temperature. The solid product was collected by suction filtration, washed with deionized water (20 mL), and air-dried.

N-(3-(1-(2-carbamothioylhydrazono)ethyl)phenyl)-1*H*-indole-2-carboxamide (**12**)

Off-white solid; yield 35%; Mp = 229-231 °C; hexane:ethyl acetate (6:4) R_f : 0.33; ^1H -NMR (500 MHz, $\text{DMSO}-d_6$) δ (ppm): 2.35 (s, 3H, CH_3), 7.09 (t, J = 7.5 Hz, 1H), 7.24 (t, J = 7.4 Hz, 1H), 7.39-7.44 (m, 2H), 7.48 (d, J = 8.2 Hz, 1H), 7.70 (d, J = 7.9 Hz, 2H), 7.79 (s, 1H), 7.95 (d, J = 7.9 Hz, 1H), 8.21 (s, 1H), 8.43 (s, 1H), 10.24 (s, 1H, N-H amide), 10.36 (s, 1H, N-H), 11.79 (s, 1H, N-H indole); ^{13}C -NMR (125 MHz, $\text{DMSO}-d_6$) δ (ppm): 14.6 (CH_3), 104.4 (CH), 112.9 (CH), 118.7 (CH), 120.4 (CH), 121.4 (CH), 122.2 (CH), 122.4 (CH), 124.3 (CH), 127.5, 129.2 (CH), 131.9, 137.3, 138.7, 139.4, 148.1 (C=N); 160.3 (C=O), 179.5 (C=S); FT-IR (KBr, cm^{-1}): 3399, 3320, 3206, 3141, 1656, 1582; HRMS ((+)-ESI): m/z =

352.12341 (calcd. 352.12321 for $C_{18}H_{18}N_5OS$ $[M+H]^+$); analytically calculated for $C_{18}H_{17}N_5OS$: C, 61.52; H, 4.88; N, 19.93. Found: C, 61.12; H, 5.20; N, 19.63%.

N-(4-(1-(2-carbamothioylhydrazono)ethyl)phenyl)-1H-indole-2-carboxamide (13)

Yellow solid; yield 63%; Mp = 292-294 °C (decomp.); hexane:ethyl acetate (6:4) R_f : 0.23; 1H -NMR (500 MHz, DMSO- d_6) δ (ppm): 2.31 (s, 3H, CH_3), 7.09 (t, J = 7.3 Hz, 1H), 7.24 (t, J = 7.4 Hz, 1H), 7.47-7.49 (2H, NH_2), 7.69 (d, J = 7.8 Hz, 1H), 7.87 (d, J = 8.4 Hz, 2H), 7.95 (s, 1H), 7.98 (d, J = 8.5 Hz, 2H), 8.27 (s, 1H), 10.19 (s, 1H), 10.33 (s, 1H, N-H amide), 11.78 (s, 1H, N-H indole); ^{13}C -NMR (125 MHz, DMSO- d_6) δ (ppm) : 14.2 (CH_3), 104.6 (CH), 112.9 (CH), 119.9 (CH), 120.4 (CH), 122.3 (CH), 124.4 (CH), 127.5, 127.7 (CH), 131.8, 133.0, 137.4, 140.5, 147.9 (C=N), 160.2 (C=O), 179.2 (C=S); FT-IR (KBr, cm^{-1}): 3409, 3346, 3283, 3166, 1652, 1599; HRMS ((+)-ESI): m/z = 352.12339 (calcd. 352.12321 for $C_{18}H_{18}N_5OS$ $[M+H]^+$); analytically calculated for $C_{18}H_{17}N_5OS$: C, 61.52; H, 4.88; N, 19.93. Found: C, 61.35; H, 5.02; N, 19.87%.

N-(3-((2-carbamothioylhydrazono)(phenyl)methyl)phenyl)-1H-indole-2-carboxamide (14)

White solid, yield 46%, Mp = 246-248 °C; hexane:ethyl acetate (1:1) R_f : 0.70; 1H -NMR (500 MHz, DMSO- d_6) δ (ppm): 7.05 (m, 4H), 7.19 (m, 3H), 7.71 (s, 1H), 7.86 (d, J = 8.8, 1H), 8.21 (s, 1H), 8.34 (s, 1H), 8.43 (s, 1H), 8.65 (s, 1H), 10.18 (s, 1H, N-H), 10.40 (s, 1H, N-H amide), 11.73 (s, 1H, N-H indole); ^{13}C -NMR (125 MHz, DMSO- d_6) δ (ppm): 104.7 (CH), 112.9 (CH), 119.6 (CH), 120.5 (CH), 122.3 (CH), 123.5 (CH), 124.5 (CH), 127.5, 128.2 (CH), 128.9 (CH), 130.4 (CH), 130.9 (CH), 131.8 (CH), 132.2, 136.7, 137.4, 139.5, 140.9, 149.7 (C=N), 160.4 (C=O), 178.4 (C=S); FT-IR (KBr, cm^{-1}): 3419, 3320, 3283, 1647, 1582; HRMS ((+)-ESI): m/z = 414.13878 (calcd. 414.13886 for $C_{23}H_{20}N_5OS$ $[M+H]^+$); analytically calculated for $C_{23}H_{19}N_5OS$: C, 66.81; H, 4.63; N, 16.94. Found: C, 66.42; H, 4.95; N 16.56%.

***N*-(4-((2-carbamothioylhydrazono)(phenyl)methyl)phenyl)-1*H*-indole-2-carboxamide (15)**

White solid; yield 80%; Mp = 219-221 °C; hexane:ethyl acetate (1:1) R_f: 0.59; ¹H-NMR (500 MHz, DMSO-*d*₆) δ (ppm): 7.05 (m, 2H), 7.21 (m, 2H), 7.31-7.48 (m, 2H), 7.59-7.72 (m, 5H), 7.82 (d, *J* = 8.9, 2H), 8.56 (s, 2H), 10.32 (s, 1H), 10.45 (s, 1H, N-H amide), 11.72 (s, 1H, N-H indole); ¹³C-NMR (125 MHz, DMSO-*d*₆) δ (ppm): 104.7 (CH), 112.9 (CH), 120.0 (CH), 121.2 (CH), 120.5 (CH), 122.3 (CH), 124.5 (CH), 126.4, 128.2 (CH), 128.8 (CH), 130.3 (CH), 131.7, 131.8, 137.5, 140.9, 149.4 (C=N), 160.3 (C=O), 178.2 (C=S); FT-IR (KBr, cm⁻¹): 3469, 3298, 3056, 1656, 1592, 1263; HRMS ((+)-ESI): *m/z* = 414.13896 (calcd. 414.13886 for C₂₃H₂₀N₅OS [M+H]⁺); analytically calculated for C₂₃H₁₉N₅OS: C, 66.81; H, 4.63; N, 16.94. Found: C, 66.43; H, 4.97; N, 17.08%.

General procedure for synthesis of 16-19

A mixture of thiosemicarbazone derivatives **12-15** (2.3 mmole), sodium acetate (11.4 mmol) and chloroacetic acid (6.4 mmol) in 20 mL ethanol was refluxed for 24 hours, then the resulted solution was cooled to room temperature, the solid product was collected by suction filtration, and washed with deionized water (20 mL).

[3-(1*H*-indol-2-yl)-8-methyl-2,4,6,7-tetraazabicyclo[7.3.1]trideca-1(13),3,5,7,9,11-hexaen-5-yl]thioacetic acid (16)

Off-white solid; yield 68%; Mp = 270-272 °C; hexane:ethyl acetate (6:4) R_f: 0.29; ¹H-NMR (500 MHz, DMSO-*d*₆) δ (ppm): 2.41 (s, 3H, CH₃), 3.90 (s, 2H, CH₂), 7.09 (t, *J* = 7.3 Hz, 1H), 7.24 (t, *J* = 7.1 Hz, 1H), 7.43-7.49 (m, 3H), 7.59 (d, *J* = 7.4 Hz, 1H), 7.69 (d, *J* = 7.8, 1H), 7.91 (d, *J* = 7.7, 1H), 8.34 (s, 1H), 10.34 (s, 1H, N-H), 11.78 (s, 1H, N-H indole), 12.00 (s, 1H, COOH); ¹³C-NMR (125 MHz, DMSO-*d*₆) δ (ppm): 15.2 (CH₃), 33.3 (CH₂), 104.5 (CH), 112.9 (CH), 118.7 (CH), 120.4 (CH), 122.0 (CH), 122.2 (CH), 124.3 (CH), 127.5 (CH), 129.2 (CH), 131.9, 137.3, 138.8, 139.5, 160.3, 160.6, 164.7, 174.4; FT-IR (KBr, cm⁻¹): 3373, 3308, 1709, 1620, 1599, 1541; HRMS ((+)-ESI): *m/z* = 392.11809 (calcd.

392.11812 for $C_{20}H_{18}N_5O_2S$ $[M+H]^+$; analytically calculated for $C_{20}H_{17}N_5O_2S$: C, 61.37; H, 4.38; N, 17.89. Found: C, 61.30; H, 4.67; N, 17.88%.

[3-(1H-indol-2-yl)-8-methyl-2,4,6,7-tetraazabicyclo[7.2.2]trideca-1(11),3,5,7,9,12-hexaen-5-yl]thioacetic acid (17)

White solid; yield 67%; Mp = 318-320 °C; hexane:ethyl acetate (6:4) R_f : 0.21; 1H -NMR (500 MHz, DMSO- d_6) δ (ppm): 2.39 (s, 3H, CH₃), 3.88 (s, 2H, CH₂), 7.09 (t, J = 7.4 Hz, 1H), 7.25 (t, J = 7.3 Hz, 1H), 7.48-7.50 (m, 2H), 7.69 (d, J = 7.9, 1H), 7.88-7.92 (m, 4H), 10.38 (s, 1H, N-H), 11.79 (s, 1H, N-H indole), 11.95 (s, 1H, COOH); ^{13}C -NMR (125 MHz, DMSO- d_6) δ (ppm): 14.9 (CH₃), 33.2 (CH₂), 104.7 (CH), 112.9 (CH), 120.0 (CH), 120.4 (CH), 122.3 (CH), 124.4 (CH), 127.5 (CH), 131.8, 133.2, 137.4, 141.0, 160.2, 160.3, 163.9, 174.4; FT-IR (KBr, cm^{-1}): 3421, 3232, 1699, 1655, 1615, 1591; HRMS ((+)-ESI): m/z = 392.11815 (calcd. 392.11812 for $C_{20}H_{18}N_5O_2S$ $[M+H]^+$); analytically calculated for $C_{20}H_{17}N_5O_2S$: C, 61.37; H, 4.38; N, 17.89. Found: C, 61.01; H, 4.48; N, 17.79%.

[3-(1H-indol-2-yl)-8-phenyl-2,4,6,7-tetraazabicyclo[7.3.1]trideca-1(13),3,5,7,9,11-hexaen-5-yl]thioacetic acid (18)

Off-white solid; yield 34%; Mp = 281-283 °C; hexane:ethyl acetate (1:1) R_f : 0.53; 1H -NMR (500 MHz, DMSO- d_6) δ (ppm): 3.83 (s, 2H, CH₂), 6.93 (d, J = 6.0 Hz, 1H), 7.02 (t, J = 6.0 Hz, 1H), 7.18 (t, J = 6.0 Hz, 1H), 7.41-7.45 (m, 6H), 7.53-7.56 (m, 2H), 7.61 (s, 1H), 7.63 (s, 1H), 7.93 (d, J = 9.0, 1H), 10.36 (s, 1H, N-H), 11.75 (s, 1H, N-H indole), 11.92 (s, 1H, COOH); ^{13}C -NMR (125 MHz, DMSO- d_6) δ (ppm): 33.3 (CH₂), 104.8 (CH), 112.9 (CH), 120.4 (CH), 120.6 (CH), 120.6 (CH), 122.3 (CH), 124.4 (CH), 124.4 (CH), 127.5, 128.3 (CH), 129.0 (CH), 129.1, 130.6 (CH), 131.8 (CH), 136.9, 137.3, 138.0, 139.5, 160.3, 161.9, 166.4, 174.5; FT-IR (KBr, cm^{-1}): 3431, 3333, 3142, 1716, 1605; HRMS ((+)-ESI): m/z = 454.13389 (calcd. 454.13377 for $C_{25}H_{20}N_5O_2S$ $[M+H]^+$); analytically calculated for $C_{25}H_{19}N_5O_2S$: C, 66.21; H, 4.22; N, 15.44. Found: C, 66.01; H, 4.35; N, 15.12%.

[3-(1H-indol-2-yl)-8-phenyl-2,4,6,7-tetraazabicyclo[7.2.2]trideca-1(11),3,5,7,9,12-hexaen-5-yl]thioacetic acid (19)

white solid, yield 50%; Mp 226-228 °C; hexane:ethyl acetate:chloroform (2:2:3) R_f : 0.37; $^1\text{H-NMR}$ (500 MHz, $\text{DMSO-}d_6$) δ (ppm): 3.84 (s, 2H CH_2), 7.05 (t, $J = 7.5$ Hz, 1H), 7.19-7.24 (m, 3H), 7.38-7.47 (m, 5H), 7.50-7.55 (m, 2H), 7.65 (d, $J = 7.8$ Hz, 1H), 7.84 (s, 1H), 7.86 (s, 1H), 10.39 (s, 1H, N-H), 11.77 (s, 1H, N-H indole); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO-}d_6$) δ (ppm): 33.3 (CH_2), 104.8 (CH), 112.9 (CH), 120.2 (CH), 120.5 (CH), 122.3 (CH), 124.4 (CH), 127.5, 128.6 (CH), 128.9 (CH), 130.3 (CH), 130.5 (CH), 131.1, 131.8, 137.4, 138.4, 139.7, 160.4, 162.0, 165.7, 174.3; FT-IR (KBr, cm^{-1}) 3362, 3272, 1714, 1653, 1590; HRMS ((+)-ESI): $m/z = 454.13369$ (calcd. 454.13377 for $\text{C}_{25}\text{H}_{20}\text{N}_5\text{O}_2\text{S}$ $[\text{M}+\text{H}]^+$); analytically calculated for $\text{C}_{25}\text{H}_{19}\text{N}_5\text{O}_2\text{S}$: C, 66.21; H, 4.22; N, 15.44. Found: C, 65.89; H, 4.45; N, 15.11%.

Biological methods

Cell line and culture conditions

The K562 leukemia cell line was cultured in RPMI (Biochrom, Berlin, Germany), whereas HCT116 and human skin fibroblast cells were cultivated in Dulbecco's modified Eagles medium (DMEM, Biochrom, Berlin, Germany). All cell lines were cultured at 37 °C and all media were supplemented with 1% of 2 mM L-glutamine (Lonza), 10% fetal calf serum (Gibco, Paisley, U.K.), 50 IU/mL penicillin/streptomycin (Sigma, St. Louis, MO), and amphotericin B (Sigma, St. Louis, MO). Cells from passage number 9-15 were used. For cytotoxicity test, the examined compounds were added at concentration ranging between 0.5-100 μM to the culture medium and incubated for 48 hours in an atmosphere of 5% CO_2 and 95 relative humidity at 37 °C.

205 *In vitro* cytotoxicity

206 *In vitro* evaluation of cytotoxicity effects associated with the examined compounds was
207 performed according to the protocol described in the ISO 10993-5 guide.¹⁶ In brief, cells were
208 seeded at a density of 8×10^3 cells per well in 96-well plates in an appropriate medium. In all
209 assays, the drugs were dissolved in DMSO immediately before the addition to cell cultures
210 and equal amounts of the solvent were added to control cells. At the end of the exposure
211 period. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Dorset,
212 UK) assay was carried out as previously described.¹⁶ The yellow tetrazolium dye of MTT
213 was reduced by metabolically active cells into an intracellular purple formazan product.
214 Absorbance values of each well were determined with a microplate enzyme-linked immuno-
215 assay (ELISA) reader equipped with a 570-nm filter. Survival rates of the controls were set to
216 represent 100% viability and untreated cultures were used as controls groups. Each
217 experiment was repeated 3 times. IC₅₀ concentrations were obtained from the dose-response
218 curves using Graph Pad Prism Software 5 (San Diego California USA, www.graphpad.com).

219 Analysis of caspase-3 enzyme activity

220 The caspase-3 colorimetric assay kit (BioVision Research Products, Milpitas, CA) was
221 used to assess changes in caspase-3 activity after treatment with the examined compounds.
222 Apoptosis was induced in treated cells before cells were collected by centrifugation at 1000
223 rpm for 10 min. Cells were lysed and supernatants were separated according to the
224 manufacture's protocol. Protein concentration in the supernatant was quantified using the
225 Bradford method. 50 µl of the reaction buffer, 200 µM of DEVD-pNA substrate were added
226 to 50 µl supernatant in a 96-well plate and incubated at 37 °C for 2 hours. After incubation,
227 the plate was read under 405 nm wavelengths using an ELISA reader (Tecan Group Ltd.,
228 Mannedorf, Switzerland).

Results and discussion

Chemistry

Reactions of **8-11** with thiosemicarbazide afforded thiosemicarbazones **12-15** as solid products in good yields (Scheme 1) *via* nucleophilic addition-elimination reaction and subsequent formation of an imine bond; these reactions were conducted in absolute ethanol under reflux and in presence of small amounts of hydrochloric acid as a catalyst. Surprisingly, reactions of **12-15** with chloroacetic acid in the presence of sodium acetate yielded the new 10(11)-membered ring compounds **16-19** in a one-pot reaction. Structures of these compounds were confirmed by IR, HRMS, and NMR (Supplementary material, Figs. S1-S4), and by elemental analysis. These data given in the experimental sections are in total agreement with the assigned structures. Moreover, the mass spectra of prepared compounds display the correct molecular ion peaks for which the measured high resolution (HRMS) data are consistent with the calculated values. DEPT experiments were employed to distinguish secondary and quaternary carbons from primary and tertiary ones.

We assume that these products are obtained by attack of the sulfur atom in **12-15** at the alpha carbon of chloroacetic acid under the mechanism of nucleophilic substitution reaction, where the chlorine atom was the leaving group. Thereafter, an intracyclization-condensation reaction was conducted, *in situ*, between the primary amino group and carbonyl of the amide in **12-15** to afford the final products **16-19**. However, the reversed sequence, i.e., the cyclization step happening prior to nucleophilic substitution might not be excluded. The methylene carbon in **16-19** resonates at about 33 ppm and is thus upfield shifted compared to its position in chloroacetic acid (*ca.* 40 ppm); this is probably due to the weaker withdrawing-inductive effect of sulfur compared to the chlorine atom. In this regard, various cyclic amidine compounds were prepared under microwave irradiation and in presence of TiCl₄

253 catalyst.¹³ Recently, we succeeded in preparing this type of cyclic amidine with benzofuran
 254 moiety instead of indole.²

255 **Scheme 1.** Conditions: (i) thiosemicarbazide, conc. HCl, EtOH, reflux, 24 h; (ii)
 256 ClCH₂COOH, AcONa, EtOH, reflux, 24 h

Product	Methyl or Phenyl
12	3-Methyl
13	4-Methyl
14	3-Phenyl
15	4-Phenyl
16	3-Methyl
17	4-Methyl
18	3-Phenyl
19	4-Phenyl

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 259

260 Biology

261 In order to examine the cytotoxic activities of compounds **12-19**, we evaluated their
 262 growth inhibition in colon cancer (HCT116), leukemia (K562), and normal human skin
 263 fibroblast cell lines. Results revealed that compounds **12**, **13** and **16-19** exhibit weak to
 264 moderate inhibition against HCT116 cell line as shown in Table 1. On the other hand, no
 265 inhibition was observed in K562 cell line, thus indicating that this scaffold selectively targets
 266 the kinase domain. Interestingly, none of compounds **12-19** exhibited toxicity in normal
 267 human fibroblasts, and thus proves that this scaffold is not toxic to normal cells. The
 268 inhibitory activity of LY294002 confirms the selectivity of **12-13** and **16-19** against HCT116
 269 and emphasizes the beneficiary effect of this series to normal cells.

Table 1. IC₅₀ values (μM) of LY294002 and **12-19** against HCT116, K562 and fibroblast cell lines.

Compound	HCT116	K562	fibroblast
	± SD ^a	± SD ^a	± SD ^a
LY294002	6.4±1	19.8±2	29.7±2
12	68±4	>300	>500
13	63±4	>300	>500
14	>300	>300	>500
15	>200	>300	>500
16	79±2	>300	>500
17	84±5	>300	>500
18	54±2	>300	>500
19	56±3	>300	>500

^aStandard deviation (SD) never exceeded 5%.

It is worth noting that attachment of the two phenyl moieties at the two sides of the carbamothioylhydrazono motif in compounds **14** and **15** led to loss of activity. This infers that steric effect might hinder the proper orientation of **14** and **15** in the kinase catalytic site and, thus in turn abolishes the inhibitory activity. Interestingly, **12**, **13** exerted relatively higher inhibitory activity against the kinase catalytic site than **14** and **15** which may confirm that steric effect hinders the proper binding interaction for open system series **12-15**.

Notably, tailoring the phenyl indole carboxamide core structure with carbamothioylhydrazono motif at positions 3 (compound **12**) or 4 (compound **13**) displayed comparable inhibitory activity. This indicates that the binding domain properly occupied the carbamothioylhydrazono motif at both positions.

On the other hand, replacing the carboxamide with carboxamidine motif and cyclization, resulted in relatively more active compounds **16-19**. This finding might be due to the adopted binding conformation in the kinase cleft that furnished better binding interaction, thus in turn increased the activity. Strikingly, **18** and **19** exhibited the highest activity among **16-19** which suggests that incorporating a phenyl moiety might push the ligand deeply in the binding site, thus increasing its binding affinity and inhibitory activity. It's worth mentioning that tailoring the phenyl indole carboxamidine at positions 3 or 4 of the phenyl moiety **18** and **19**, respectively, did not affect the binding mode and inhibitory activity. This confirms that the proper binding conformations of **18** and **19** are derived by steric effect.

Remarkably, incorporating the thioacetic acid functionality in compounds **16-19** might have a significant role in their inhibitory activity; this might be due to the ionic and/or H-bond interaction supplied by the carboxylic acid group. In addition, the thioether linkage provided flexibility which facilitates access of the ligand deep in the binding domain. Additionally, swapping the carboxamide with carboxamidine motif might provide an ionic and/or H-bond interaction thus increasing the binding affinity and inhibitory activity. Though the difference between the carboxamide and carboxamidine motifs is replacing O with N atom while both atoms provide H-bond acceptor; the ionic interaction might enhance the water solubility and thus providing an extra polar surface area. Altogether, the steric effect as well as the carboxamidine and thioacetic acid linkage rationalize the relative high potency of **16-19**.

In order to identify whether growth inhibition of colon cancer cell line was mediated by an apoptotic pathway, we assessed the caspase-3 enzyme activity using the caspase-3 colorimetric assay. Initiation of caspase-3 pathway¹⁴⁻¹⁵ is a characteristic of apoptosis and can be employed in cellular assays to evaluate activators and inhibitors of the “death cascade.” To verify the apoptotic effects of the examined compounds on HCT, cells were incubated with either 0.1 mM or 0.2 mM of each compound for 48 h and changes in caspase-3 activities were detected and measured. Results showed that there is a substantial increase in caspase-3 activity in a dose-response manner for each tested compound. Indeed, increasing the concentration of the examined compound resulted in further increase in caspase-3 activity relative to the untreated cells control (Fig. 3). Compound **18** was the most potent HCT116 inhibitor and apoptosis inducer among prepared molecules.

Fig. 3. Influence of compounds treatment on caspase 3 activity in HCT116 cells after 48 h. The results are the means of two independent experiments. $P < 0.05$ was considered significant.

Conclusion

We have successfully prepared and characterized a new series of thiosemicarbazone-based indole derivatives and 3-(indol-2-yl)bicyclotetrazatridecahexaens. Biological results showed that these compounds selectively exert cytotoxic activity on HCT116 cell line and none of them exhibited any toxicity on K562 and normal human skin fibroblast. Our future goal is to optimize the core structure of this series to induce its cytotoxic activity and selectivity against a panel of cancer cell lines.

Supplementary material

^1H NMR and ^{13}C NMR data of **16-19** are available with the article through the journal Web site.

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Graphical Abstract

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