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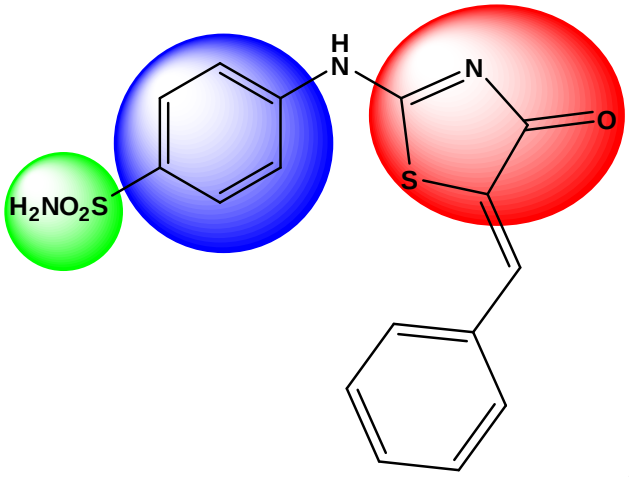

3a

IC_{50} = 0.334 µM

Caspase-3 conc. = 0.43 nmol/mL

Fas-ligand conc. = 775.2 pg/mL

^{99m}Tc-compound **3a** complex conc. = 16.02±2.43 %ID/g at 120min

Design, Synthesis and Biological Evaluation of Some Novel Sulfonamide Derivatives as Apoptosis Inducers

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Abstract

Several novel thiazolidinone and fused thiazolidinone derivatives bearing benzenesulfonamide moiety were synthesized and confirmed *via* spectral and elemental analyses. The newly synthesized compounds were evaluated for their cytotoxic activity on colorectal cancer cell line (Caco-2). All the synthesized compounds showed better activity than the reference standards (Doxorubicin and 5-FU). Investigation of the apoptotic activity of the most active compounds revealed that compounds **3a, 5a, 5c** and **6c** activate both caspase-3 and Fas-ligand in Caco-2 cell line. Compound **3a** was the most active compound with caspase-3 concentration of 0.43 nmol/mL and Fas-ligand concentration of 775.2 pg/mL in treated Caco-2 cells. Compound **3a** was radiolabeled with ^{99m}Tc and its biodistribution pattern was evaluated *in vivo* using normal Swiss Albino mice. ^{99m}Tc-compound **3a** complex didn't exhibit any accumulation in any body organs except for its accumulation in the colon; target organ; where it showed 8.97±1.35 %ID/g at 15min p.i. that elevated till 16.02±2.43 %ID/g at 120min p.i.

Keywords: Thiazolidinone, Caspase-3, Fas-ligand, Apoptosis, Radiolabeling, Technetium-99m

1. Introduction

Cancer is one of the most challenging diseases to human race being one of the highest mortality rates all over the world. Colorectal cancer comes in the third place after lung and breast cancer invading more than a million patients every year with more than 60% mortality rate. Men suffer more from colorectal cancer and its incidence is increased with age in developing countries [1]. Age, Lifestyle and genetic disorders are on the top of its risk factors, besides lack of physical activity, smoking, alcohol, obesity, diabetes, exposure to radiation and family history [2]. The choice of proper treatment depends on the patient health condition and the goal of treatment. Similar to all types of cancer; surgery, radiation and chemotherapy are used to manage the condition with limitations and side effects [3].

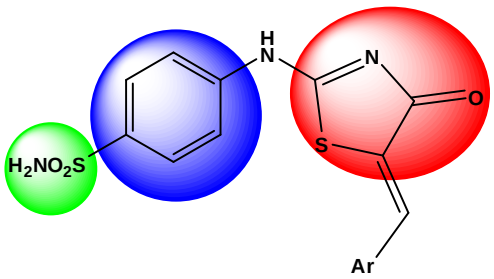
Aryl sulfonamide derivatives have received great attention as anticancer agents through the past decade [4-11]. Several sulfonamide derivatives have been evaluated as anticancer agents and proven to exhibit good activity through many mechanisms including their action as carbonic anhydrase inhibitors [12-14], metalloprotienase inhibitors [15], NADPH reductase inhibitors [16], histone deacetylase inhibitors [17], PI3K inhibitors [18] and microtubules assembly disruption [19].

On the other hand, several 4-thiazolidinone was a versatile synthone for many derivatives with a wide range of biological activities [20-30]. Among these activities 4-thiazolidinones and its arylidene derivatives comprise a major class in the search for new

anticancer agents against many types of cancer as breast cancer cell line (MCF-7) [31], JNK stimulatingphosphatase-1 (JSP-1) [32], tumor necrosis factor (TNFa) [33], antiapoptotic biocomplex (Bcl-XL-BH3) [34], integrin avb3 receptor [35], CDK1/Cyclin B inhibition [36] and coloncancer (HT29) [37]. Also fused heterocylic thiazole derivatives especially fused pyrimidine derivatives exhibited good anticancer activity [38, 39]. Structure activity relationship studies on several thiazolo[4,5-d]pyrimidines have shown good anticancer activity for those derivatives with an aryl substitution on number 7 [40]. Moreover, thiazolo[4,5-d]pyrimidines were evaluated for their apoptotic activity through caspase-3 [41].

Furthermore, one of the major pathways for apoptosis induction in cells is the Fas/Fas-ligand (FasL) system. In a similar way to cytolytic activity of tumor necrosis factor, Fas is a component of the cell surface that has cell-killing activity [42, 43]. Its corresponding ligand (FasL) is a membrane protein [44]. Activation of Fas by its natural ligand (FasL) or an antibody transmits a death signal to the cells and cause apoptosis. Despite the widespread of Fas in both normal and cancer cells, FasL is limited to macrophages, natural killers and T activated cell [45, 46]. After cellular activation FasL protein exists in intracellular stores [47]. Engagement of the cell death surface receptor Fas by Fas ligand (FasL) results in apoptotic cell death, mediated by caspase activation [48].

Based on the above facts and as a continuation of our search for new anticancer agents, several benzylidinethiazolidinone derivatives bearing benzenesulfonamide moiety were synthesized, thiazolopyrimidine & thioxopyrimidine derivatives and thiazoloisoxazole derivatives were cyclized from their arylidine intermediates (Figure 1). All the newly synthesized compounds were evaluated *in vitro* for their cytotoxic activity against human colon cancer cell line (Caco-2 cell line). The most active compounds were further evaluated for their apoptotic activity through increase of caspase-3 and Fas & FasL (Fas ligand) in Caco-2 cell line cells treated with the newly synthesized compounds. Moreover, biodistribution for the most active compound was evaluated through ^{99m}Tc-compound complex radiolabeling to determine its *in vivo* pharmacokinetic behavior and its body organs uptakes besides its elimination pathway.



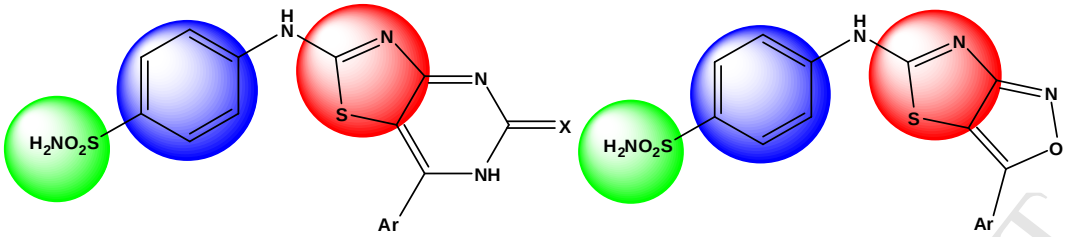


Figure 1: General Structures for the newly synthesized compounds

2. Results and Discussion

2.1. Chemistry

Benzenesulfonamide thiazolidinone derivative **2** was synthesized from 2-chloro-N-(4-sulfamoylphenyl)acetamide **1** through cyclization with ammonium thiocyanate [50]. The structure of the formed compound was verified by its elemental and spectral analyses. ^{1}H-NMR spectrum in (DMSO-d_6) of **2** exhibited a singlet signal at δ: 3.98 ppm corresponding to CH_2 of thiazolidinone and a singlet signal at δ: 11.86 ppm attributed to NH as it was exchanged upon treatment with D_2O. Arylidene derivatives **3 a-c** were prepared by reaction of the active methylene group of thiazolidinone with different aromatic aldehydes in the presence of catalytic amounts of anhydrous sodium acetate. The structures of these derivatives were confirmed *via* elemental and spectral analyses.

^{1}H-NMR spectra in (DMSO-d_6) of **3 a-c** exhibited singlet signals for C=CH of arylidines in the range of δ: 7.87-8.01 ppm. Reaction of arylidene intermdiates **3 a-c** with urea or thiourea and KOH afforded the cyclized thiazolo[4,5-d]pyrimidines **4 a-c** & **5 a-c**, respectively. The structures of the synthesized compounds were verified by their ^{1}H-NMR spectra which exhibited singlet signals for NH of pyrimidine in the range of δ: 3.33-3.40 ppm while singlet signals corresponding to NH attached to thiazole moiety were in the range of δ: 11.96-12.24 ppm. These signals were exchanged upon treatment with D_2O. Finally, thiazoloisoxazole derivatives **6 a-c** were prepared by reacting aryiledene derivatives **3 a-c** with hydroxylamine and KOH. The structures of the synthesized compounds were verified by their ^{1}H-NMR that showed increased aromatic protons (**Scheme 1**).

2.2. Cytotoxic assay

Cytotoxicity of the newly synthesized compounds was studied against Caco-2 tumor cell line using the colorimetric methyl tetrazolium test (MTT) as described and modified by Mosmann [49]. Percentage of relative viability and the half maximal inhibitory concentration IC_{50} was calculated by the prism program Version 6 (**Table 1**).

From the results, it became clear that the newly synthesized compounds have significant effect on Caco-2 cell line for 24 hrs. Viability test by MTT Assay using treatment of Caco-2 cell line showed that all the synthesized compounds were much more active than the reference standards (Doxorubicin, IC_{50}= 63.30 µM and 5-Fluorouracil, IC_{50}= 31.00 µM) The activity of the newly synthesized compounds can be arranged in a descending order as follows: compounds **5a**, **5c** and **6c** were most effective on cell line viability and caused inhibition of cell growth, with IC_{50} value of 0.333 µM followed by compounds **3a** and **4a** with IC_{50} value of 0.334 µM. The activity almost drops to its half in case of compound **6b** IC_{50} value of 0.559 µM followed by compounds **3c**, **4b** and **5b** with IC_{50} value of 0.667 µM with almost the same activity of compound **6a** with IC_{50} value of 0.669 µM. On the other hand, compound **3b** showed higher IC50 value of 1.33 µM while compound 4c was the least active compound with IC_{50} value of 12.70 µM.

Structure Activity Relationship

On a further look on **Table 1,** we can categorize the newly synthesized compounds in the light of their biological results to study the relationship between chemical structure and biological effect on Caco-2 cell line in some points.

Firstly, it seems that thiazolothioxopyrimidine derivatives **5a-c** is the best series in activity compared to others (arylidene derivatives **3a-c**, thiazolopyrimidinone derrivatives **4a-c** and thiazoloisoxazole derivatives **6a-c**). This can be concluded from the IC_{50} values of the compounds belong to this series. Two of the most active four compounds, **5a** and **5c**, are members of this series with IC_{50} value of 0.333 µM. The activity tends to be lower in case of thiazoloisoxazole series **6a-c** followed by arylidene derivatives **3a-c** and finally the lowest activity was attributed to the thiazolopyrimidinone series **4a-c**.

Secondly, the effect of *para* substitution on the phenyl ring was variable among different series. In case of arylidene derivatives **3a-c**, the unsubstituted derivative 3a was the most active compound with IC_{50} value of 0.334 µM. This activity drops to its half in case of *p*-Cl derivative **3c** and getting much worse in case of *p*-OCH_3 derivative **3b**.

In case of thiazolopyrimidinone derivatives **4a-c**, again the unsubstituted derivative **4a** was the most active compound with IC_{50} value of 0.333 µM. However in this series the chloro derivative **4c** was least active compound with relatively high IC_{50} value of 12.7 µM. This was not the case in their thioxo nalouges **5a-c**, as both the unsubstituted and the chloro derivatives **5a** and **5c** showed the same activity with almost double the activity of methoxy derivative **5b**. In the last series, thiazoloisoxazole derivatives **6a-c**, the chloro derivative was the most active compound with IC_{50} value of 0.333 µM followed by the methoxy derivative **6b** and the unsubstituted derivative **6a**.

Finally, comparing the isosteric series, thiazolopyrimidinone derivatives **4a-c** & thiazolothioxopyrimidine derivatives **5a-c**, lead to the assumption of that thioxo

derivatives are better derivatives for cytotoxic activity despite of the same IC_{50} values for the unsubstituted and methoxy analogues, the chloro derivative of thioxo series was almost 40 times more active than its oxo analogue.

2.3. Fas ligand Assay

In order to investigate the apoptotic behavior for the most active compounds in cytotoxic assay **3a, 5a, 5c** and **6c**, Fas ligand concentration in CaCo 2 cell line was measured by using the colorimetric human factor-Related Apoptosis Ligand (FASL) and results were displayed in **Table 2**.

All the tested compounds showed significant increase in the concentration of Fas-ligand relative to negative control and better than the reference standards (doxorubicin and 5-FU) supporting the assumption of the apoptotic activity of the synthesized compounds. Compound **3a** was also the most active compound in this assay with Fas-ligand concentration of 775.2 pg/mL.

2.4. Caspase-3 Assay

In exploring the apoptotic activity of the newly synthesized compounds, the most active compounds in cytotoxic assay **3a, 5a, 5c** and **6c** were subjected to caspase-3 assay. The caspase-3 activities were measured in Caco-2 cell line treated with drugs, doxorubicin and 5-FU as positive control. All compounds showed significant increase in the caspase-3 activity of Caco-2 cell line compared to negative control and reference drugs as shown in **Table 3**.

The increment of caspase-3 concentration was significant for all compounds relative to control and positive control drug (doxorubicin) but less than that produced by 5-FU. Compound **3a** was also the most active compound in this assay with caspasce-3 concentration of 0.43 nmol/mL.

2.5. Radiolabeling and Biodidtribution

*2.5.1. Preparation of ^{99m}Tc-compound **3a** complex*

As presented in **Figure 2 (A-D)**, studying four different factors optimized the preparation of ^{99m}Tc-compound **3a** complex; reducing agent ($NaBH_4$) amount, compound **3a** amount, pH and the reaction time. The highest radiochemical yield of ^{99m}Tc-compound **3a** complex was 85.2±0.4% that was obtained using reaction condition; 100 µl $^{99m}TcO_4^-$(~200 MBq), 15 mg of $NaBH_4$, 0.5 mg of compound 3a, pH 9 and the reaction mixtures were kept at room temperature for 30 min.

2.5.2. Biological pattern of ^{99m}Tc-compound 3a complex

The biodistribution compartments as (average percent-injected dose per gram) of ^{99m}Tc-compound **3a** complex are illustrated in **Figure 3.** As reflected in this figure**,** high uptake was in the kidney at 15min p.i., which dropped regularly indicating that the main clearance route of ^{99m}Tc-compound 3a complex was mainly *via* renal pathway. ^{99m}Tc-compound **3a** complex didn't exhibit any accumulation pattern in any body organ except for its accumulation in the colon, which was at 15min p.i. It showed 8.97±1.35 %ID/g and elevated till 16.02±2.43 %ID/g at 120min p.i.

Conclusion

In light of the biological results, we can conclude that compounds **3a, 5a, 5c** and **6c** are good cytotoxic agents compared to the reference standards (doxorubicin and 5-FU). The cytotoxic activity of these compounds is attributed to their apoptotic activity through activation of caspase-3 and Fas-ligand in Caco-2 cell line. Further biological investigation should be conducted for other mechanisms of apoptosis. Moreover, compound **3a** is accumulated selectively in colon with high renal elimination rate that may contribute to future investigation for its selectivity for colon cancer treatment.

3. Experimental

3.1. Chemistry

Melting points were determined on Electro thermal Stuart 5MP3 digital melting point apparatus and were uncorrected. Elemental microanalyses were performed at the micro analytical centre, Al-Azhar University, Cairo, Egypt. IR spectra were recorded on a Bruker Fourier transform (FT)- IR spectrophotometer as KBr discs. NMR spectra (in DMSO-d6) were recorded on Bruker AC-300 Ultra Shield NMR spectrometer (Bruker, Flawil, Switzerland, δ ppm) at 400 MHz using TMS as internal Standard and peak multiplicities are designed as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Silica gel used for column chromatography was obtained from Fluka, 70—230 mesh thin layer chromatography was carried out on silica gel TLC plates with fluorescence indicator (F_{254}).

3.1.1. *4-((4-Oxo-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide* **2**

A solution of 2-chloro-N-(4-sulfamoylphenyl) acetamide **1** (2.625g, 10 mmol) and ammonium thiocyanate (0.76 gm, 10 mmol) in absolute ethanol (20 mL) was refluxed for 3 hours and the obtained precipitate during reflux was filtered, washed with water, dried and recrystallized from ethanol, yield 70 %, m.p.250- 252 ºC. IR (KBr) cm^{-1}: 3323, 3246, 3205 (NH), 3051 (arom. CH), 2995, 2929 (aliph. CH), 1683 (C=O), 1635, 1604 (C=C, C=N).^{1}H NMR (DMSO-d_6, D_2O) δ: 3.98 (s, 2H, CH_2-S), 7.11-7.48 (m, 6H, arom.CH,

NH_2SO_2, D_2O-exchangeable), 11.86 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 34.42 (C5, thiazole), 119.95 (C3, benzenesulphonamide), 121.32 (C5, benzenesulphonamide), 127.00 (C2, 6, benzenesulphonamide), 139.59 (C1, 4-benzenesulphonamide), 150.90 (C2, thiazole), 174.14 (C4, thiazole, C=O). Anal. Calc. for $C_9H_9N_3O_3S_2$ (271). Calculated: C, 39.85; H, 3.32; N, 15.49. Found: C, 40.04; H, 3.37; N, 15.72%.

3.1.2. 4-((5-Arylidene-4-oxo-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide **3a-c**

A mixture of 4-(2-imino-4-oxothiazolidin-3-yl)benzenesulfonamide **2** (2.71g, 10 mmol), appropriate aromatic aldehyde (10 mmol) and anhydrous sodium acetate (0.82 gm, 10 mmol) in glacial acetic acid (15 mL) was refluxed for 12 hours then allowed to cool. The solid product was collected, dried and recrystallized from acetic acid.

3.1.2.1. 4-((5-Benzylidene-4-oxo-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide **3a**

Yield 57 %, m.p.265- 267 ºC. IR (KBr) cm^{-1}: 3358, 3269, 3253 (NH), 3049 (arom. CH), 1676 (C=O), 1620, 1604 (C=C, C=N). ^1H NMR (DMSO-d_6, D_2O) δ: 7.29 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.11-7.83 (m, 9H, arom.CH), 7.87 (s, 1H, C=CH), 11.96 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 120.20 (C3, benzenesulphonamide), 120.94 (C5, benzenesulphonamide), 121.46 (C4, benzene), 126.94 (C2, 6, benzene), 127.21 (C5, thiazole), 129.21 (C3, 5, benzene), 129.63.14 (C2, 6, benzenesulphonamide), 129.91 (C1, benzene), 139.61 (C1, 4, benzenesulphonamide), 140.05 (methylenecarbon), 152.00 (C2, thiazole), 174(C4, thiazole, C=O). Anal. Calc. for $C_{16}H_{13}N_3O_3S_2$ (359). Calculated: C, 53.48; H, 3.62; N, 11.69. Found: C, 53.66; H, 3.66; N, 11.85%.

3.1.2.2.*4-((5-(4-Methoxybenzylidene-4-oxo-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide* **3b**

Yield 65 %, m.p.278- 280 ºC. IR (KBr) cm^{-1}: 3325, 3250, 3100 (NH), 3050 (arom. CH), 2956 (CH aliph), 1670 (C=O), 1637, 1595 (C=C, C=N). ^1H NMR (DMSO-d_6, D_2O) δ: 3.84 (s, 3H, OCH_3),7.34 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.06-7.97 (m, 8H,arom.CH), 8.00 (s, 1H, C=CH), 12.20 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 55.82 (OCH3), 115.15 (C3, 5, benzenesulphonamide), 121.96 (C1,benzene), 126.86 (C3,5,benzene), 128.99 (C2,6,benzene), 131.96 (C2, 6, benzenesulphonamide), 132.63 (C1, benzenesulphonamide), 133.74, (C5, thiazole), 139.95 (C4, benzenesulphonamide), 144.77 (methylenecarbon), 160.96 (C4, benzene),161.58 (C2, thiazole), 165.30 (C4, thiazole, C=O). Anal. Calc. for

$C_{17}H_{15}N_3O_4S_2$ (359). Calculated: C, 52.44; H, 3.85; N, 10.79. Found: C, 52.67; H, 3.41; N, 11.04%.

3.1.2.3.4-((5-(4-Chlorobenzylidene-4-oxo-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide **3c**

Yield 68 %, m.p. >300 ºC. IR (KBr) cm^{-1}: 3325, 3250, 3201 (NH), 3045 (arom. CH), 1670 (C=O), 1635, 1589 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 7.30 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.18-7.99 (m, 8H, arom.CH), 8.01 (s, 1H, C=CH), 12.47 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 120.59 (C3, benzenesulphonamide), 126.57 (C5, benzenesulphonamide), 127.25 (C1, benzene), 128.62 (C3, 5, benzene), 129.27 (C2, 6, benzene), 131.27 (C2, 6, benzenesulphonamide), 131.71 (C1, benzenesulphonamide, C5, thiazole), 131.80 (C4, benzenesulphonamide), 134.49 (methylenecarbon), 140.18 (C4, benzene), 164.75 (C2, thiazole), 166.46 (C4, thiazole, C=O). Anal. Calc. for $C_{16}H_{12}ClN_3O_3S_2$ (393.5). Calculated: C, 48.79; H, 3.60; N, 10.67. Found: C, 49.02; H, 3.69; N, 10.79%.

3.1.3.4-((5-Oxo-7-aryl-5,6-dihydrothiazolo[4,5-d]pyrimidin-2-yl)amino)benzenesulfonamide **4a-c**

A mixture of 0.01 mol of the required chalcone **3a-c** (0.01 mol), urea (0.6g, 0.01mol) and KOH (0.56g, 0.01 mol) in 20 mL ethanol was heated under reflux for 6 hours, then cooled and poured onto crushed ice. The obtained solid product was filtered and recrystallized from ethanol.

3.1.3.1.4-((5-Oxo-7-phenyl-5,6-dihydrothiazolo[4,5-d]pyrimidin-2-yl)amino)benzenesulfonamide **4a**

Yield 65 %, m.p. 273-275 ºC. IR (KBr) cm^{-1}: 3360, 3317, 3201 (NH), 3049 (arom. CH), 1676 (C=O), 1637, 1602 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 3.35 (s, 1H, NH, D_2O-exchangeable), 7.29 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.12-7.83 (m, 9H, arom.CH), 11.96 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 120.15 (C7a, thiazolopyrimidine), 121.47 (C3, benzenesulphonamide), 127.00 (C5, benzenesulphonamide), 127.20 (C3, 5, benzene), 129.22 (C2, 6, benzene), 129.64 (C2, 6, benzenesulphonamide), 129.93 (C4, benzene), 139.61 (C1, benzenesulphonamide, C1, benzene), 140.09 (C4, benzenesulphonamide), 147.47 (C7, thiazolopyrimidine), 150.79 (C2, thiazolopyrimidine), 157.65 (C3a, thiazolopyrimidine), 174.00 (C5, thiazolopyrimidine, C=O). Anal. Calc. for $C_{17}H_{13}N_5O_3S_2$ (399). Calculated: C, 51.12; H, 3.25; N, 17.54. Found: C, 51.38; H, 3.22; N, 17.71%.

3.1.3.2.4-((5-Oxo-7-(4-methoxyphenyl)-5,6-dihydrothiazolo[4,5-d]pyrimidin-2-yl)amino)benzenesulfonamide **4b**

Yield 73 %, m.p. > 300 ºC. IR (KBr) cm^{-1}: 3336, 3250, 31200 (NH), 3070 (arom. CH), 1668 (C=O), 1637, 1593 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 3.33 (s, 1H, NH, D_2O- exchangeable), 3.78(s, 3H, OCH_3), 7.34 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.06-7.87 (m, 8H, arom.CH), 12.24 (s, 1H, NH, D_2O-exchangeable).^{13}C NMR (DMSO-d_6) δ: 55.37 (OCH_3), 114.81 (C3, 5, benzenesulphonamide), 119.80 (C7a, thiazolopyrimidine), 120.55 (C2, 6, benzenesulphonamide), 121.49 (C3, 5, benzene), 125.68 (C1, benzenesulphonamide), 127.20 (C2, 6, benzene), 129.94 (C1, benzene), 131.63 (C4, benzenesulphonamide), 140.01 (C7, thiazolopyrimidine), 144.60 (C4, benzene), 151.45 (C2, thiazolopyrimidine), 160.62 (C3a, thiazolopyrimidine), 168.50 (C5, thiazolopyrimidine, C=O). Anal. Calc. for $C_{18}H_{15}N_5O_4S_2$ (429). Calculated: C, 50.34; H, 3.49; N, 16.31. Found: C, 50.49; H,3.51; N, 16.54%.

3.1.3.3.4-((5-Oxo-7-(4-chlorophenyl)-5,6-dihydrothiazolo[4,5-d]pyrimidin-2-yl)amino)benzenesulfonamide **4c**

Yield 67 %, m.p. >300 ºC. IR (KBr) cm^{-1}: 3327, 3250, 3200 (NH), 3050 (arom. CH), 1674 (C=O), 1630, 1589 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 3.36 (s, 1H, NH, D_2O- exchangeable), 7.35 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.19-7.86 (m, 8H, arom.CH), 12.20 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 120.45 (C7a, thiazolopyrimidine), 121.45 (C3, 5, benzenesulphonamide), 123.44 (C1, benzenesulphonamide), 127.22 (C2, 6, benzene), 128.52 (C1, benzene), 129.23 (C3, 5, benzene), 131.23 (C2, 6, benzenesulphonamide), 132.04 (C4, benzene), 132.75 (C7, thiazolopyrimidine), 134.45 (C4, benzenesulphonamide), 140.14 (C2, thiazolopyrimidine), 150.54 (C3a, thiazolopyrimidine), 168.00 (C5, thiazolopyrimidine, C=O). Anal. Calc. for $C_{17}H_{12}ClN_5O_3S_2$ (433.5). Calculated: C, 47.05; H, 2.76; N, 16.14. Found: C, 47.36; H, 2.79; N, 16.38%.

3.1.4.4-((7-Aryl-5-thioxo-5,6-dihydrothiazolo[4,5-d]pyrimidin-2-yl)amino)benzenesulfonamide **5a-c**

Chalcone derivatives **3a-c** (0.01 mol) thiourea (0.02 mol, 1.52 g) and KOH (0.02 mol, 1.12 g) were taken in a 100 mL round bottom flask. To the above reaction mixture ethanol (30 mL) were added. Reaction mixture was refluxed for 6 hrs using water condenser. It was then cooled and poured in cold water. Acidified with dil HCl, filtered washed with water and dried. The product was recrystallization from ethanol to get the product **5a-c**.

3.1.4.1.4-((7-phenyl-5-thioxo-5,6-dihydrothiazolo[4,5-d]pyrimidin-2-yl)amino)benzenesulfonamide **5a**

Yield 70 %, m.p. 268-270 ºC. IR (KBr) cm^{-1}: 3360, 3317, 3271 (NH), 3049 (arom. CH), 1637, 1602 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 3.33 (s, 1H, NH, D_2O-exchangeable),7.34 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.11-7.86 (m, 9H, arom.CH), 12.11 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 117.16 (C7a, thiazolopyrimidine), 120.40 (C4, benzene), 120.76 (C3, 5, benzenesulphonamide), 126.54 (C3, 5, benzene), 127.51 (C2, 6, benzene), 130.29 (C1, benzenesulphonamide), 132.51 (C1, benzene), 138.76 (C2, 6, benzenesulphonamide), 140.09 (C4, benzenesulphonamide), 149.36 (C7, thiazolopyrimidine), 152.50 (C2, thiazolopyrimidine), 157.82 (C3a, thiazolopyrimidine), 181.01 (C5, thiazolopyrimidine, C=S). Anal. Calc. for $C_{17}H_{13}N_5O_2S_3$ (415). Calculated: C, 49.15; H, 3.13; N, 16.86. Found: C, 49.37; H, 3.19; N, 17.08%.

3.1.4.2.4-((7-(4-Methoxyphenyl)-5-thioxo-5,6-dihydrothiazolo[4,5-d]pyrimidin-2-yl)amino)benzenesulfonamide **5b**

Yield 65 %, m.p. >300 ºC. IR (KBr) cm^{-1}: 3340, 3251, 3199 (NH), 3047 (arom. CH), 1639, 1593 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 3.34 (s, 1H, NH, D_2O-exchangeable), 3.77 (s, 3H, OCH_3), 7.34 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.105-7.87 (m, 8H, arom.CH), 12.20 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 55.35 (OCH_3), 114.80 (C3, 5, benzenesulphonamide), 119.44 (C7a, thiazolopyrimidine), 120.11 (C3, benzene), 121.48 (C2, 6, benzene), 125.67 (C5, benzene), 127.67 (C2, 6, benzenesulphonamide), 129.96 (C1, benzene), 131.62 (C1, 4, benzenesulphonamide), 139.62 (C4, benzene), 140.00 (C7, thiazolopyrimidine), 152.00 (C2, thiazolopyrimidine), 160.61 (C3a, thiazolopyrimidine), 179.45 (C5, thiazolopyrimidine, C=S). Anal. Calc. for $C_{18}H_{15}N_5O_3S_3$ (445). Calculated: C, 48.53; H, 3.37; N, 15.73. Found: C, 48.69; H, 3.43; N, 15.49%.

3.1.4.3.4-((7-(4-Chlorophenyl)-5-thioxo-5,6-dihydrothiazolo[4,5-d]pyrimidin-2-yl)amino)benzenesulfonamide **5c**

Yield 72 %, m.p. >300 ºC. IR (KBr) cm^{-1}: 3325, 3250, 3203 (NH), 3047 (arom. CH), 1637, 1597 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 3.40 (s, 1H, NH, D_2O-exchangeable), 7.35 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.18-7.86 (m, 8H, arom.CH), 12.20 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 120.53 (C7a, thiazolopyrimidine), 121.48 (C3, benzenesulphonamide), 122.00 (C5, benzenesulphonamide), 127.24 (C2, 6, benzene), 128.52 (C1, benzenesulphonamide), 129.27 (C3, 5, benzene), 131.27 (C2, 6, benzenesulphonamide), 132.01 (C1, benzene), 134.46 (C4, benzene, C4, benzenesulphonamide), 140.15 (C7, thiazolopyrimidine),

151.35 (C2, thiazolopyrimidine), 156.58 (C3a, thiazolopyrimidine), 179.15 (C5, thiazolopyrimidine, C=S). Anal. Calc. for $C_{17}H_{12}N_5O_2S_3$ (449.5). Calculated: C, 45.38; H, 2.66; N, 15.57. Found: C, 45.52; H, 2.64; N, 15.81%.

3.1.5. 4-((3-Arylthiazolo[4,5-c]isoxazol-5-yl)amino)benzenesulfonamide **6a-c**

To a mixture of chalcone derivatives **3a-c** (0.01 mol) and hydroxylamine hydrochloride (0.7 g , 0.01 mol) in ethanol (10 mL) a solution of KOH (0.56 g , 0.01 mol) in ethanol (50 mL) was added. The reaction mixture was refluxed for 6 h and then kept overnight at room temperature. After cooling in ice, the reaction mixture was acidified with aqueous HCl (10%). The resulting precipitate was washed with distilled water and dried. The resulting crude was crystallized from ethanol to obtained products **6a-c**.

3.1.5.1. 4-((3-phenylthiazolo[4,5-c]isoxazol-5-yl)amino)benzenesulfonamide **6a**

Yield 73 %, m.p. >300 ºC. IR (KBr) cm^{-1}: 3315, 3246, 3200 (NH), 3050 (arom. CH), 1631, 1595 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 7.35 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.19-7.87 (m, 9H, arom.CH), 12.33 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 120.79 (C3a, thiazoloisoxazole), 121.81 (C4, benzene), 121.99 (C3, 5, benzenesulphonamide), 127.56 (C2, 6, benzene), 129.53 (C3, 5, benzene), 129.97 (C2, 6, benzenesulphonamide), 130.26 (C1, benzene, C1, benzenesulphonamide), 131.24 (C6a, thiazoloisoxazole), 133.53 (C4, benzenesulphonamide), 140.44 (C3, thiazoloisoxazole), 169.00 (C5, thiazoloisoxazole). Anal. Calc. for $C_{16}H_{12}N_4O_3S_2$ (372). Calculated: C, 51.61; H, 3.22; N, 15.05. Found: C, 51.85; H, 3.26; N, 15.23%.

3.1.5.2. 4-((3-(4-Methoxyphenyl)thiazolo[4,5-c]isoxazol-5-yl)amino)benzenesulfonamide **6b**

Yield 63 %, m.p. >300 ºC. IR (KBr) cm^{-1}: 3338, 3250, 3225 (NH), 3050 (arom. CH), 1637, 1593 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 3.77 (s, 3H, OCH_3) 7.46 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.05-7.87 (m, 8H, arom.CH), 12.22 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 55.35 (OCH_3), 114.79 (C3, 5, benzenesulphonamide), 119.46 (C3a, thiazoloisoxazole), 120.35 (C1, benzene), 121.48 (C3, 5, benzene), 125.65 (C1, benzenesulphonamide), 127.19 (C2, 6, benzene), 129.96 (C6a, thiazoloisoxazole), 131.61 (C2, 6, benzenesulphonamide), 139.99 (C4, benzenesulphonamide), 150.81 (C4, benzene), 151.93 (C3, thiazoloisoxazole), 167.59 (C5, thiazoloisoxazole). Anal. Calc. for $C_{17}H_{14}N_4O_4S_2$ (402). Calculated: C, 50.74; H, 3.48; N, 13.93. Found: C, 51.01; H, 3.54; N, 14.19%.

3.1.5.3. 4-((3-(4-Chlorophenyl)thiazolo[4,5-c]isoxazol-5-yl)amino)benzenesulfonamide **6c**

Yield 70 %, m.p. >300 ºC. IR (KBr) cm^{-1}: 3325, 3250, 3200 (NH), 3047 (arom. CH), 1637, 1589 (C=C, C=N). ^{1}H NMR (DMSO-d_6, D_2O) δ: 7.35 (s, 2H, NH_2SO_2, D_2O-exchangeable), 7.20-7.86 (m, 8H, arom.CH), 12.35 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6) δ: 120.45 (C3a, thiazoloisoxazole), 120.57 (C3, benzenesulphonamide), 121.45 (C5, benzenesulphonamide), 123.45 (C2, benzene), 127.25 (C6, benzene), 128.53 (C3, 5, benzene), 129.27 (C1, benzene, C1, benzenesulphonamide), 131.27 (C2, 6, benzenesulphonamide), 132.06 (C4, benzene), 134.48 (C6a, thiazoloisoxazole), 140.16 (C4, benzenesulphonamide), 150.55 (C3, thiazoloisoxazole), 167.38 (C5, thiazoloisoxazole). Anal. Calc. for $C_{16}H_{11}$ Cl $N_4O_3S_2$ (406.5). Calculated: C, 47.29; H, 2.70; N, 13.79. Found: C, 47.38; H, 2.68; N, 13.98%.

3.2. In vitro cytotoxicity test by MTT assay

3.2.1. Cell lines

Cancer colon cell line (Caco-2) was obtained frozen in liquid nitrogen (-180 ºC) from the American Type Culture Collection. The cell lines were maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Cells were cultured in RPMI-1640 medium (RPMI-1640, Sigma–Aldrich, USA). The medium was supplemented with antibiotic-free 10% fetal bovine serum (FBS, Sigma, USA), 100 U/mL penicillin and 2-mg/mL streptomycin. The cells were sub cultivated after trypsination (Trypsin-EDTA, Cambrex, BioScience Verviers, Belgium) once or twice per week and resuspended in complete medium in a 1:5 split ratio. Cell lines were maintained as monolayer in T75 cell culture flasks with filter screw caps (TPP, Trasadingen, Switzerland) at 37 °C in a humidified 5% CO_2 incubator.

3.2.2. Cell viability analysis by Trypan blue:

Cells on each panel were cultured in 24-well plates and incubated for 24 hr. Cells treated with gradual concentrations ranges (100 μM to 1μM) of each drug then cells incubated for 24hr and the viability was examined using trypan blue dye by light microscopy.

3.2.3. Cytotoxicity analysis by MTT assay

Cytotoxicity of compounds were studied against Caco-2 tumor cell lines and using the colorimetric methyl tetrazolium test (MTT) as described and modified by *Mosmann 1983* [49]. Percentage of relative viability and the half maximal inhibitory concentration (IC_{50}) was calculated by the prism program (Graph Pad prim 7)..

3.3. Measurement of Human Factor –Related Apoptosis Ligand (FASL) activity

The Fas/Fas-ligand (Fas-L) system has been recognized as a major pathway for the induction of apoptosis in cells and tissues**.** Fas- ligand concentration in Caco-2 cell line was measured by using the colorimetric Human Factor-Related Apoptosis Ligand (FASL) ELISA kit **(WKEA MED SUPPLIES company , China) (Code No.WH-174)** after incubation Caco-2 cell line with drugs **3a, 5a, 5c** and **6c** and positive slandered drugs (Doxorubicin & 5-Fluorouracil) at different concentrations for 24 hrs.

3.4. Measurements of Caspase-3 enzymatic activity

Apoptosis is mediated by proteolytic enzymes, which called Caspases, which trigger cell death; Caspase-3 is an "effector" caspase associated with the initiation of the "death cascade" and is therefore an important marker of the cell's entry point into the apoptotic signaling pathway. The caspase-3 activity was measured by using **colorimetric Bender Med System Caspase 3 assay kit; Cat No: BMS2012INST** after incubation Caco-2 cell line with drugs **3a, 5a, 5c** and **6c** and positive slandered drugs (Doxorubicin & 5-Fluorouracil at different concentrations for 24 hrs.

3.5. Radiolabeling and Biodistribution

3.5.1. Chemicals and Equipment

Reactive grade chemicals and reagents needed were bought from Sigma-Aldrich Chemical Company and Merck Company. We chose purged deoxygenated bidistilled water to use in radiolabeling procedure. Technetium-99m was eluted in the form of $^{99m}TcO_4^-$ from $^{99}Mo/^{99m}Tc$ generator, Gentech, Turkey. A good-type NaI scintillation γ-Counter model scalar ratemeter SR7 (Nuclear Enterprises Ltd., USA) and Shimadzu reversed phase-HPLC that is composed of pumps LC-9A, Rheodyne injector, UV spectrophotometer detector (SPD-6A) operated at a wavelength of 254 nm and a reversed-phase column (RP-18, 250 x4.6 mm, 5μm, Lichrosorb) were used.

3.5.2. Animal model

In accordance with the Egyptian Atomic Energy Authority (EAEA), Animal studies were executed and it was also asserted by Animal ethics committee. Animal models used in the *in vivo* experiment were Normal Swiss albino mice of body mass range 25-50g and were purchased from Helwan University, Egypt. Mice were maintained in a cabin of size that suits groups of five in the room temperature with a 12h light/dark cycle and accessibility to food and water.

*3.5.3. Preparation of ^{99m}Tc-compound **3a** complex*

One mL DMSO was used as a solvent for Compound **3a** (0.5mg) in 10 mL penicillin vials. Exactly 15 mg of $NaBH_4$ was then added to each vial. 0.1 N Sodium Hydroxide was used to maintain in the pH range of 7-11. Freshly prepared 100 μL $^{99m}TcO_4^-$(~200

MBq) was then added to each of these vials. Duration of these reactions was about 30 minutes at room temperature. Different $NaBH_4$ amounts (5-25 mg), compound **3a** amounts (0.25-4 mg), within different time periods (5-240 min) were repeatedly used in the aforementioned procedure to reevaluate the radiochemical yields.

*3.5.4. Radiochemical yield assay of ^{99m}Tc-compound **3a** complex*

Strips of ascending Whatman paper chromatography (PC) and HPLC were used to assess radiochemical yield and *in vitro* stability of ^{99m}Tc-compound 3a complex. For PC, two strips were used for each procedure, on each strip, 2 drops of the reaction product were placed were on origin line 2 cm far from the base. To determine percentage of free $^{99m}TcO_4^-$ radio-contaminant, the developing solvent for one PC strip was acetone, where free $^{99m}TcO_4^-$ R_f was 1 whereas ^{99m}Tc- compound 3a complex and reduced hydrolyzed technetium colloid species R_f was zero. Another strip was developed in C_2H_5OH: H_2O: NH_4OH mixture (2:5:1,v/v/v) to define the ratio of the hydrolyzed ^{99m}Tc radio-contaminant, where reduced hydrolyzed technetium colloid R_f is zero while free $^{99m}TcO_4^-$ and ^{99m}Tc- compound 3a complex species R_f is 1. As the developing process was ending, the strips were dried, trimmed into 1 cm size pieces and counted using the sodium iodide (Tl) γ-ray scintillation counter.

The following equation was followed to calculate the radiochemical yield percent of ^{99m}Tc- compound 3a complex:

$$\%\,\text{Radiochemical yield} = 100 - (\%\,\text{Free}\ ^{99m}TcO_4^- + \%\,\text{colloid})$$

More affirmation was to be gained using Shimadzu HPLC as an extra mean to evaluate the radiochemical yields. The sample mixtures (50 µL) were injected into a reversed-phase column (Lichrospher RP18, 4 mm X 250 mm; 5 µm). The HPLC was run at a wavelength of 254 nm using mobile phase of acetonitrile: water (15: 85, v/v) with flow rate adjusted to 1 mL/min. Then portions of 0.5 mL were separated distinctly using a fraction collector up to 20 mL and counted in a good-type γ-scintillation counter.

*3.5.5. Biological distribution of ^{99m}Tc-compound **3a** complex*

Guidelines of both Egyptian Atomic Energy Authority and The animal ethics committee were followed. Pharmacokinetic and distribution studies were done in Normal Albino mice. ^{99m}Tc- compound 3a complex biodistribution was evaluated at time periods of 15, 60 and 120 min post injection (p. i) in normal Albino mice (n = 5 mice/time point). Mice were housed in groups and nourished with food and water. ^{99m}Tc- compound 3a complex was injected in the mice subcutaneously. Primarily, Chloroform was used to anaesthetize mice, then weighted and sacrificed at different time periods. All body organs and tissues were detached, collected and washed with saline then weighted. Specimens of blood, bone and muscles were collected and weighted then were assumed to be 7, 10 and 40 %

of the overall body weight, respectively [51-55]. A good type γ-counter NaI(Tl) was used to measure radioactivities of both organs and the background. The percent-injected dose/gram organ or fluid (% ID/g) was calculated in aggregates of five.

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Figure & Table Captions

Figure 1: General Structures for the newly synthesized compounds

Fig. 2. Factors affecting radiochemical yield of ^{99m}Tc-compound **3a** complex.

Fig. 3. Biodistribution pattern of ^{99m}Tc-compound 3a complex

Scheme 1. Synthesis of compounds **1-6**

Table 1.

In vitro anticancer screening of the synthesized compounds against colon cancer cell line (Caco-2).

Table 2.

Fas-ligand concentration in treated Caco-2 cell line.

Table 3.

Caspase-3 activity in treated Caco-2 cell line

Table 1.

In vitro anticancer screening of the synthesized compounds against colon cancer cell line (Caco-2).

Compound	Compound concentration (µM)					IC_{50} (µM)
	1µM	10µM	25µM	50µM	100µM	
	Surviving fraction					
3a	0.348	0.182	0.133	0.422	0.068	0.334
3b	0.281	0.179	0.466	0.416	0.485	1.33
3c	0.285	0.314	0.113	0.086	0.153	0.667
4a	0.371	0.237	0.285	0.229	0.227	0.334
4b	0.477	0.362	0.473	0.257	0.167	0.667
4c	0.519	0.603	0.112	0.156	0.073	12.70
5a	0.304	0.223	0.234	0.223	0.135	0.333
5b	0.444	0.344	0.299	0.186	0.169	0.667
5c	0.433	0.456	0.338	0.339	0.276	0.333
6a	0.442	0.335	0.304	0.295	0.456	0.669
6b	0.412	0.401	0.317	0.229	0.228	0.559
6c	0.319	0.248	0.219	0.202	0.197	0.333
Doxorubicin	0.814	0.597	0.663	0.552	0.343	63.30
5-FU	0.405	0.310	0.325	0.525	0.254	31.00

Table 2.

Fas-ligand concentration in treated Caco-2 cell line.

Compound	Concentration (µM)	Concentration of Fas – ligand (pg/mL)
Control	0	20.5
3a	0.15	445.2
	0.3	775.2
	0.6	706.12
5a	0.15	115.5
	0.3	165.7
	0.6	307.5
5c	0.15	312.4
	0.3	309.4
	0.6	641.8
6c	0.15	111.3
	0.3	147.5
	0.6	312.2
Doxorubicin	31.5	41.65
	63.5	56.41
	100	95.8
5-FU	62	66.2
	31	140.1
	15	287.7

Table 3.

Caspase-3 activity in treated Caco-2 cell line

Compound	Concentration (µM)	Caspase-3 concentration (nmol/mL)
Control	0	0.193
3a	0.6	0.37
	0.3	0.38
	0.15	0.43
5a	0.6	0.364
	0.3	0.34
	0.15	0.35
5c	0.6	0.37
	0.3	0.366
	0.15	0.404
6c	0.6	0.407
	0.3	0.409
	0.15	0.366
Doxorubicin	31	0.251
	62.3	0.26
	100	0.272
5-FU	31	0.8
	15	0.92
	62	0.73

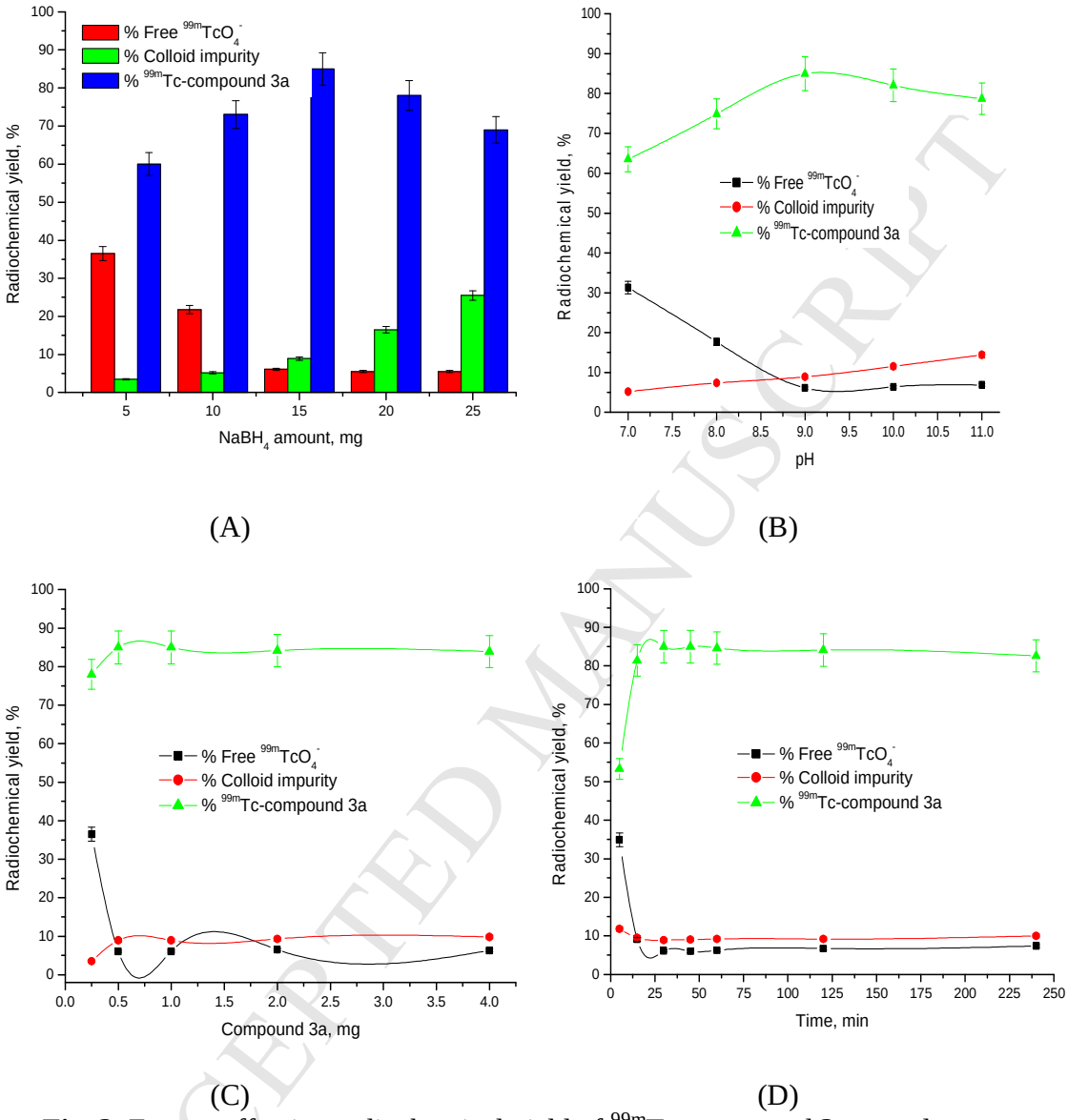


Fig. 2. Factors affecting radiochemical yield of ^{99m}Tc-compound **3a** complex.

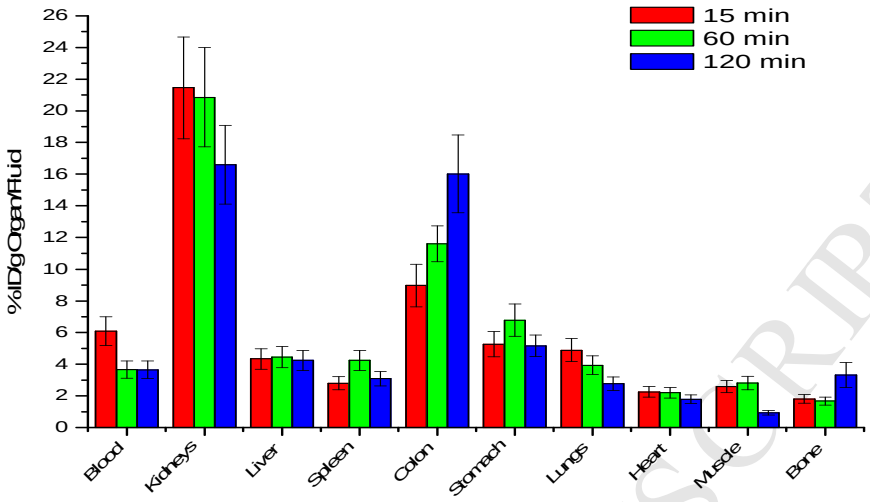
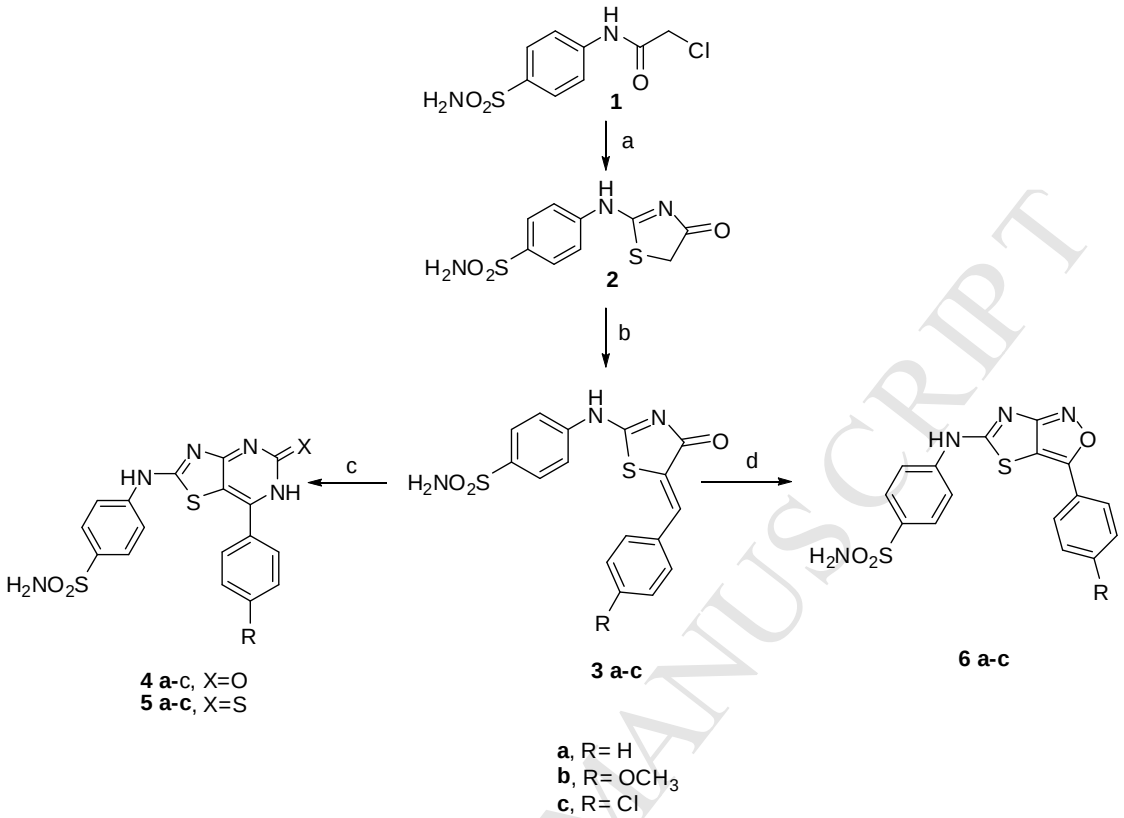


Fig. 3. Biodistribution pattern of ^{99m}Tc-compound 3a complex



Scheme 1. Synthesis of compounds **1-6**

- Novel thiazolidinone and fused thiazolidinone derivatives.
- *In vitro* cytotoxic activity on colorectal cancer cell line (Caco-2).
- *In vitro* activation of Caspase-3 and Fas-ligand in Caco-2 cell line.
- Radiolabeling with ^{99m}Tc and biodistribution pattern was evaluated *in vivo.*