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Elaborating 5-(4-Chlorophenyl)-1,3,4-Thiadiazole Scaffold with A *P*-Tolyl Sulfonamide Moiety Enhances Cytotoxic Activity: Design, Synthesis, *in Vitro* Cytotoxicity Evaluation, Radiolabelling and *in Vivo* Pharmacokinetic Study

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Abstract

Novel 1,3,4-thiadiazole derivatives (3-5) were designed, synthesized, and screened for cytotoxic activity. Compound 3 bearing substituted benzenesulfonamide scaffold exhibited remarkable potency against breast cancer (MCF-7), hepatoma (HepG2), colon cancer (HCT116), and lung cancer (A549) cells and lower potency on the normal cells (WI-38), as well as, it possesses higher anticancer activity than starting 5-(4-chlorophenyl)-1,3,4-thiadiazole-2-amine (2) and the positive control Staurosporine. The anticancer activity of compound 3 is at least partly attributed to the inhibition of the tumor-associated human carbonic anhydrase isoforms IX and XII. The high selectivity of compound 3 for cancer cells over normal cells and for tumor-associated CA isoforms IX and XII over the off-target cytosolic isoform II reflects its safety. Pharmacokinetic study of compound 3 was evaluated in a normal mice model based on a radiopharmaceutical chemistry approach.

Keywords:1,3,4-thiadiazoles; sulfonamides; carbonic anhydrase inhibitors; anticancer activity; radiolabeling, in vivo pharmacokinetics.

1. Introduction

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or metastasize to other parts of the body causing death. Worldwide, cancer is the second leading cause of death after heart disease accounting for nearly 10 million deaths in 2020 [1]. Lung, colon, rectum, liver, stomach, and breast cancers are the most dangerous cancer types responsible for mortality. The use of non-selective chemotherapeutic agents puts the patients at high risk of serious side effects as they affect both cancer and normal rapidly dividing cells [2]. This situation provokes medicinal scientists to find new selective anticancer agents.

1,3,4-Thiadiazole is one of the key five-membered sulfur-containing heterocyclic scaffolds. The sulfur-

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containing heterocycles have diverse chemical features due to the presence of electron-deficient and bivalent sulfur atom with two areas of positive electrostatic potential, which can interact with electron donors such as oxygen and nitrogen atoms[3,4]. 1,3,4-Thiadiazoles are attractive scaffolds in drug design due to their ability for non-covalent interactions involving sulfur atom and H-bonds involving nitrogen atoms [4]. On basis of previously reported work, it was revealed that 1,3,4-thiadiazole-containing compounds treat different cancer types through variable mechanisms of action (Figure 1) [5]. Filanesib (I) is a kinesin spindle protein (KSP) inhibitor used in clinical trials for patients with multiple myeloma, advanced/refractory myeloid leukemia, and advanced solid tumors [6]. Compound II synthesized by Zhao Jie et al (2014) showed potent antiproliferative activity against MCF-7 (breast cancer) and A549 (lung cancer) cell lines with IC50 values of 1.12 and 1.25 µM, respectively [7]. Furthermore, compound III exhibited maximum HDAC inhibitory activity with an IC50= 0.018 µM against HDAC-1 and an IC50= 0.31 µM in the HCT-116 cell proliferation assay [8].

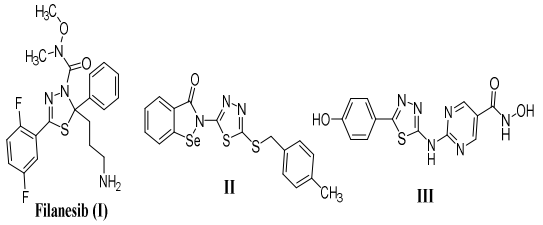


Figure 1: Chemical structure of 1,3,4-thiadiazole containing agents I-III showing antiproliferative activity

Sulfonamide is another essential class of compounds that possesses a wide range of pharmacological activities. Recently, structurally novel compounds featuring aromatic/heterocyclic sulfonamide skeleton have been reported to show substantial antitumor activity in vitro and/or in vivo (Figure 2) [9]. Among these compounds, SLC-0111 (IV), one of the most advanced potent anticancer sulfonamides, has completed phase II and bearing now phase Ib/II clinical trials for the treatment of advanced hypoxic solid tumors [10]. Moreover, the diaryl sulfonamide V displayed broad-spectrum antiproliferative activity against different cancer types; leukemia (SR), melanoma (SK-MEL-5), and breast cancer (T-47D and MDA-MB-468) with % growth inhibition ranges of 81.58%, 84.32%, 90.47%, and 84.83%, respectively at 10 µM [11]. Also, the substituted sulfonamide VI showed high anticancer potential against human gastric adenocarcinoma (AGS cells) and human colorectal adenocarcinoma (HT-29 cells) with IC50 values of 4.0 µM and 4.4 µM, respectively [12].

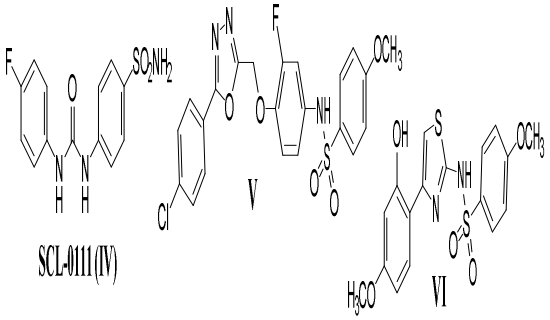


Figure 2: Chemical structure of sulfonamide containing anticancer agents IV-VI

Sulfonamides act as antitumor agents through variable mechanisms of action, one of these mechanisms is carbonic anhydrase (CA) inhibition [2,8]. Carbonic anhydrases (CAs) are abundant zinc enzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate by a metal hydroxide nucleophilic mechanism. CAs are essential in numerous physiological and pathological processes, including tumorigenesis. To date, 15 different isoforms of human CAs (hCAs) have been identified with different catalytic activities, cellular and subcellular localization, and tissue distributions. Recently, at least two tumor-associated CA isozymes have been identified (CA IX and CA XII). CA IX shows restricted expression in normal tissues but is tightly associated with different types of tumors, mostly due to its strong induction by tumor hypoxia. CA XII is present in various normal tissues and overexpressed in some tumors. Therefore, selective inhibition of hCA IX and XII in cancer cells emerged as an attractive therapeutic target in the development of anticancer agents [13].

Primary sulfonamides like acetazolamide (AZA) VII (Figure 3), broadly inhibit different CA isoforms through coordination with zinc ion in the CA active site resulting in several side effects [2]. According to Baglini et al. (2021), secondary sulfonamides IX have been recently reported as efficient and selective inhibitors of the cancer-related hCA IX and XII isoforms. In comparison to primary sulfonamides

VIII, they showed greater selectivity for the tumor-associated isoforms over the ubiquitous isoform CA II (Figure 3) [13].

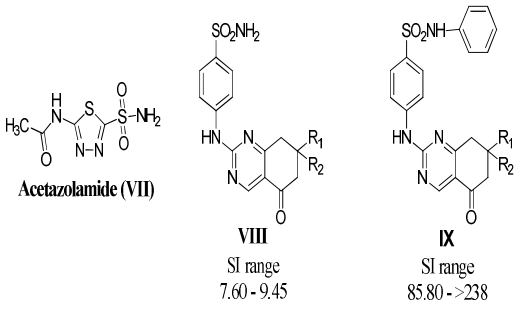


Figure 3: Chemical structure of carbonic anhydrase (CA) inhibitors VII-IX.

Based on the aforementioned information, we aimed to study the effect of merging the two anticancer pharmacophores, namely 5-aryl-1,3,4-thiadiazole and the secondary sulfonamide on the anticancer potency and selectivity of the designed compound. Hence, this study presented the synthesis and cytotoxic activity assessment of N-(5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl)-4-methylbenzene sulfonamide (3). The new compound 3 is expected to have enhanced cytotoxicity than the parent 5-(4-chlorophenyl)-1,3,4-thiadiazole-2-amine. For comparative reasons, the effect of converting the 1,3,4-thiadiazole ring into a bicyclic thiadiazolopyrimidinone system in compound 4 or grafting a benzoyl acetamide moiety in compound 5, on cytotoxic activity of thiadiazole 2 was also studied, In addition, carbonic anhydrase inhibitory potential of 3 was evaluated against CA I, II, IX, and XII isoforms. Finally, the pharmacokinetic behavior of compound 3 was studied with the aid of radiolabeling technique to evaluate its accumulation in different organs.

2. Experimental procedures:

2.1. Materials and methods

2.1.1. *Apparatus*. Melting points were obtained using a Stuart melting point apparatus and were uncorrected. Microanalyses for C, H, and N were performed at the Regional Center for Mycology and Biotechnology, Al-Azhar University. IR spectra were recorded on Shimadzu IR 435 spectrophotometer (Shimadzu Corp., Kyoto, Japan) at the Faculty of Pharmacy, 6 October University for Modern Sciences and Arts (MSA University), Giza, Egypt, and values are presented in cm-1. 1H NMR spectra were recorded using a Bruker 400 MHz (Bruker Corp., Billerica, MA, USA) spectrophotometer at the Faculty of Pharmacy, Cairo University, Cairo, Egypt. Tetramethylsilane (TMS) was used as an internal-standard, and chemical shifts were recorded in ppm on the δ scale and coupling constants (J) were reported in Hz. 13C NMR spectra were recorded using a Bruker 100 MHz spectrophotometer at the Faculty of Pharmacy, Cairo University, Cairo, Egypt. The progress of the reactions was monitored with TLC using precoated aluminum sheet silica gel MERCK 60F 254. The spots were visualized using a UV lamp. The eluent system used was ethyl acetate: hexane [3:7]. p-Chlorobenzaldehyde and thiosemicarbazide were purchased from Sigma Aldrich.

2.1.2. Synthesis of 2-(4-chlorobenzylidene) hydrazine-1-carbothioamide (1) [14]

p-Clorobenzaldehyde (14.06 g, 0.1 M) in warm alcohol (150 mL) and thiosemicarbazide (9.11 g, 0.1 M) in warm water (150 mL) were mixed slowly with continuous stirring. The product was separated immediately on cooling, filtered, dried, and recrystallized from ethanol.

Eluent: n-hexane/ethyl acetate (70/30 v/v). Product 18.53 g was separated as white crystals, yield 87%. mp 205-207 °C (reported mp 207 °C).

2.1.3. Synthesis of 5-(4-chlorophenyl)-1,3,4-thiadiazol-2-amine (2) [14]

Compound 1 (10.56 g, 0.05 M) was suspended in 300 ml warm water, and FeCl3 (24.30 g, 0.15 M) in 300 ml water was added quantitatively, slowly with constant stirring. The contents were heated at 80-90 °C for 45 min. The solution was filtered hot and then citric acid (21.10 g, 0.11 M) and sodium citrate (12.90 g, 0.05 M) were added. The resulting mixture was divided into 4 parts and each part was neutralized separately with ammonia (10%). The required amine was separated out, filtered, dried, and recrystallized from ethanol.

Eluent: n-hexane/ethyl acetate (70/30 v/v). Product 7.11 g was separated as buff crystals, yield 68%. mp 225-227 °C (reported mp 227); IR (KBr, vmax cm-1): 3272, 3090 (NH_2 forked peak, 1H NMR (DMSO- d6, 400 MHz) δ: 7.47 (s, 2H, NH_2, D_2O exchangeable), 7.51 (d, 2H, J=8.40 Hz, ArH, 4-chlorophenyl), 7.76 (d, 2H, J=8.40 Hz, ArH, 4-chlorophenyl).

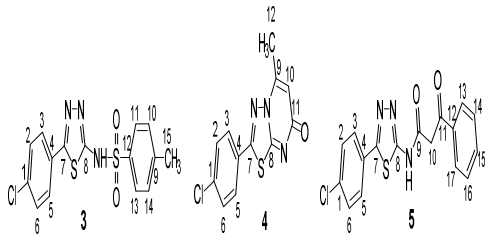


Figure 4: Carbon atoms numbering for ^{13}C elucidation of compounds 3-5

2.1.4. Synthesis of N-(5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl)-4-methylbenzene sulfonamide (3)
Compound 2 (0.21 g, 1 mmol) was refluxed with p-tolylsulfonyl chloride (0.57 g, 3 mmol) in dry pyridine (5 mL) for 3 h. The progress of the reaction was monitored by TLC. The reaction mixture was poured on dil. HCl solution and the formed precipitate was filtered, washed with distilled water, dried, and crystallized from ethanol.
Eluent: n-hexane/ethyl acetate (70/30 v/v). Product 0.30 g was separated as brown crystals, yield 83%. mp 260-262 °C; IR (KBr, νmax cm-1): 3447 (NH), 1595, 1550 (C=N), 1320, 1151 (SO_2), 1H NMR (DMSO- d_6, 400 MHz) δ: 2.37 (s, 3H, CH3), 7.36 (d, 2H, J=8.20 Hz, ArH, 4-tolyl), 7.58 (d, 2H, J=8.56 Hz, ArH, 4-chlorophenyl), 7.73 (d, 2H, J=8.20 Hz, ArH, 4-tolyl), 7.84 (d, 2H, J=8.56 Hz, ArH, 4-chlorophenyl), 14.47 (s, 1H, NH, D2O exchangeable). 13C NMR (DMSO d6, 100 MHz) δ: 21.49 (C-15), 126.16 (C-11,13), 127.70 (C-3,5), 128.12 (C-10,14), 129.45 (C-2,6), 129.48 (C-4), 136.94 (C-1), 139.12 (C-12), 143.04 (C-9), 154.37 (C-8), 166.92 (C-7). Anal. Calcd. For $C_{15}H_{12}ClN_3O_2S_2$ (365.85): % C, 49.25; H, 3.31; N, 11.49; found: % C, 49.41; H, 3.49; N, 11.72.

2.1.5. Synthesis of 2-(4-Chlorophenyl)-5-methyl-7H-[1,3,4]thiadiazolo[3,2- a]pyrimidin-7-one (4)
A mixture of compound 2 (0.21 g, 1 mmol) and ethyl acetoacetate (0.39 g, 3 mmol) were fused at a temperature of 250 oC in a sand bath for 12 hrs. The formed precipitate was triturated with cold ethanol and crystallized from methanol.
Eluent: n-hexane/ethyl acetate (70/30 v/v). Product 0.20 g was separated as black crystals, yield 72%, m.p. 287-289 °C; IR (KBr, νmax cm-1): 3051, 3032 (CH aromatic), 2917 (CH aliphatic), 1694 (C=O), 1660 (C=N), 1595 (C=C); 1H NMR (DMSO- d6, 400 MHz) δ: 2.31 (s, 3H, CH3), 6.33 (s, 1H, ArH, pyrimidinone), 7.68 (d, 2H, J=8.40 Hz, ArH, 4-chlorophenyl), 7.98 (d, 2H, J=8.40 Hz, ArH, 4-chlorophenyl). 13C NMR (DMSO-d_6, 100 MHz) δ: 23.80 (C-12), 107.38 (C-10), 127.56 (C-4), 129.59 (C-2,6), 130.20 (C-3,5), 138.04 (C-1), 156.45 (C-7), 157.34 (C-8), 161.70 (C-9), 163.55 (C-11). Anal. Calcd. For C12H8ClN3OS (277.73): C, 51.90; H, 2.90; N, 15.13; found: C, 52.13; H, 3.06; N, 15.40.

2.1.6. Synthesis of N-(5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl)-3-oxo-3-phenylpropanamide (5)
A mixture of compound 2 (0.21 g, 1 mmol) and ethyl benzoylacetate (0.57 g, 3 mmol) were fused at a temperature of 250 oC in a sand bath for 12 h. The formed precipitate was triturated with cold ethanol and crystallized from methanol.
Eluent: n-hexane/ethyl acetate (70/30 v/v). Product 0.27 g was separated as pale brown crystals, yield 76%), m.p. 212-214 °C; IR (KBr, νmax cm-1): 3058 (CH aromatic), 2876 (CH aliphatic), 1650 (C=O), 1610-1600 (C=N), 1580 (C=C); 1H NMR (DMSO-d6, 400 MHz) δ: 4.41 (s, 2H, CH2), 7.51-7.60 (m, 5H, ArH, phenyl), 7.97-8.02 (m, 4H, ArH, 4-chlorophenyl), 12.89, 13.34 (2s, 1H, NH/OH, D2O exchangeable). 13C NMR (DMSO-d6, 100 MHz) δ: 47.21 (C10), 126.27 (C-14), 128.38 (C-16), 128.82 (C-13), 129.12 (C-17), 129.38 (C-3,5), 129.91 (C-2,6), 134.41 (C-4), 135.73 (C-15), 135.27 (C-1), 149.37 (C-12), 158.98 (C-8), 161.49 (C-7), 166.66 (C-9), 194.36 (C-11). Anal. Calcd. For $C_{17}H_{12}ClN_3O_2S$ (357.81): C, 57.07; H, 3.38; N, 11.74; found: C, 57.31; H, 3.50; N, 11.98.

2.2. Cytotoxicity screening

Anticancer activity was carried out at the Confirmatory Unit, VACSERA, Cairo, Egypt. The in vitro anticancer activity of the newly synthesized compounds was evaluated against MCF-7, HepG2, HCT116, PC3, A549, and WI-38 employing Staurosporine as the positive control according to the MTT method. The MTT system is a method for measuring the activity of living cells through mitochondria dehydrogenases. MTT assay is based on using (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl tetrazoliumbromide) or MTT which is water soluble yellow dye. Upon adding MTT to viable cells, mitochondrial dehydrogenases cleave the tetrazolium solution, thus they are dissolved in acidified isopropanol to yield a purple solution. The purple solution was measured spectrophotometrically to estimate the toxicity degree caused by the test material [15–20].

2.2.1. Cell culture protocol. Breast (MCF-7), hepatoma (HepG2), colon (HCt116), prostate (PC3), lung (A549), and normal (WI-38) cells were obtained from American Type Culture Collection, cells were cultured using Dulbecco's modified Eagle's medium

(DMEM) (Invitrogen/Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 10 mg/ml of insulin (Sigma-Aldrich), and 1% penicillin-streptomycin. All of the other chemicals and reagents were purchased from Sigma-Aldrich or Invitrogen. The culture medium was transferred to a centrifuge tube. To remove any traces of serum, the cell layer was washed with 0.25% (w/v) trypsin in 0.53 mM EDTA solution. Trypsin EDTA solution 2.0-3.0 mL was added and cells were examined under an inverted microscope until the cell layer was dispersed (5-15 min). Complete growth medium 6.0-8.0 mL was added and cells were aspirated by gentle pipetting. The cell suspension in addition to the medium and cells from the previous step was centrifuged (5-10 min) at 125×g. The supernatant was thrown out then a fresh growth medium was added to the cell pellet and the cell suspension was transferred to new culture vessels. Cultures were incubated for 24 h at 37 °C. Cells were treated with serial concentrations of the test compounds and Staurosporine then incubated for 48 h at 37 °C then proceeded for the MTT assay.

2.2.2. MTT cytotoxic assay protocol. Cells were plated in a volume of 100 ml complete growth medium (cells density 1.2-1.8×10,000 cells/well) and 100 ml of the tested compound per well in a 96-well plate for 24 h before the MTT assay. Cultures from the incubator were removed into a laminar flow hood or other sterile work areas. Each vial of MTT [M-5655] to be used was reconstituted with 3 mL of medium or balanced salt solution without phenol red and serum. Reconstituted MTT was added in an amount equal to 10% of the culture medium volume. Cultures were incubated for 2-4 h depending on cell type and maximum cell density. MTT Solubilization Solution [M-8910] was added to cultures to dissolve the resulting formazan crystals and dissolution was enhanced by mixing in a gyratory shaker. Moreover, trituration was helpful for complete dissolution. ROBONIK P2000 was used to measure the color intensity at a wavelength of 450 nm. To draw the survival curve for MCF-7, HepG2, HCT116, PC3, A549, and WI-38 cell lines after a specified time, the surviving fraction was plotted versus the drug concentration. The half maximal inhibitory concentration (IC50) was calculated for the test compounds and the positive control drug Staurosporine. The surviving fractions were expressed as means ± S.E.M.

2.3. Carbonic anhydrase enzyme inhibition assay

The enzyme inhibition assays of human Carbonic anhydrase (hCA) isoforms I, II, IX, and XII were performed at the Neurofarba Department, University of Florence, Italy. To determine the activity of CA-mediated CO2 hydration, an Applied Photophysics stopped-flow instrument has been utilized. The absorbance (λmax; 557 nm) of the color intensity obtained from a solution containing indicator 0.2 mM (phenol red), buffer 20 mM (Hepes) to maintain pH 7.5, and Na2SO4 20 mM was measured to determine the initial rates of the CA-catalyzed CO_2 hydration reaction. To analyze the kinetic parameters and inhibition constants, the CO2 concentrations were adjusted between 1.7 and 17 mM. To calculate the initial velocity, six traces of the first 5-10% of the reaction progress were measured for each inhibitor. Determination of the uncatalyzed rates was carried out by employing the same procedures and deducted from the total rates. The stock solution of the tested compound (0.01 mM) was dissolved in distilled-deionized water and then diluted with the assay buffer to 0.01 nM. The formation of the E-I complex was reached by incubating the tested compound and the enzyme at room temperature or 4 °C before the assay. Enzyme and inhibitor were incubated for 15 min then data were collected. PRISM 3 was utilized to measure the mean from three determinations of inhibition constants. All CA isoforms were recombinant ones obtained in Florence [21–28].

2.4. Radiolabeling and in vivo bio-distribution of radio-iodinated compound 3

2.4.1. Synthesis of radio-iodinated compound 3:

The electrophilic substitution method was used to radio-iodinate compound 3 and its radiosynthesis yield was assessed by ascending paper chromatography (P.C.) and TLC as reported [29–32]. Optimization of radiosynthesis yield was attained via the full study of each factor as follows: pH [3-10]; Chloramine-T amount [100-500 µg]; Compound 3 amount [1-12 µg] and Reaction time [5-60 min].

2.4.2. Radioactive in-vivo tracing studies of radio-iodinated compound 3 using normal mice model:

Egyptian Atomic Energy Authority had approved through the animal ethics committee the animal studies regulation for performing all preclinical studies and all its regulations were followed intensively. Normal female mice (20-25 g) were used. All the biodistribution screening protocols followed the reported protocol [33–37]. Percent-injected dose per organ (% ID/organ ± S.D.) at each time point for a population of five mice were reported. One-way ANOVA test was used to evaluate data differences ($P<0.05$).

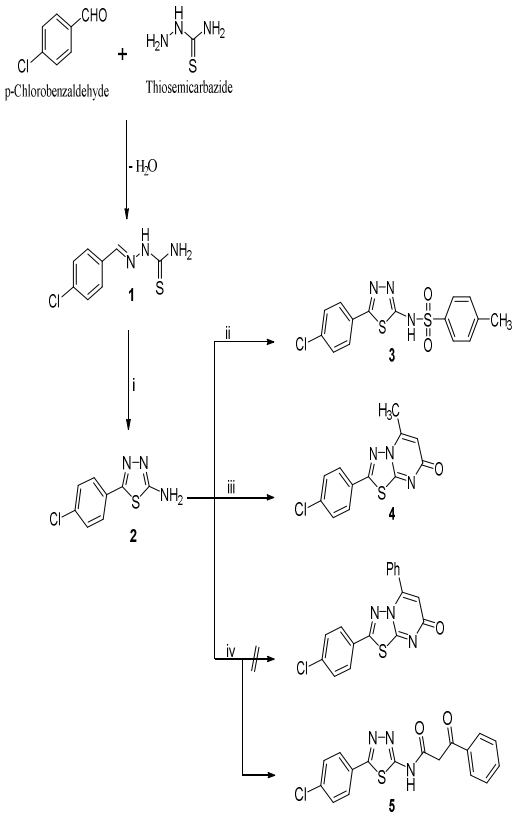
3. Results and Discussion:

3.1. Chemistry

The starting compound 5-(4-chlorophenyl)-1,3,4-thiadiazol-2-amine (2) was obtained by the oxidative cyclization of thiosemicarbazone intermediate (1) (formed by condensation of p-chlorobenzaldehyde and thiosemicarbazide) in the presence of ferric chloride according to reported procedures.

The reaction of compound 2 with p-tolylsulfonyl chloride in dry pyridine produced the target compound N-(5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl)-4-methylbenzene sulfonamide (3), as shown in Scheme 1. The structure of compound 3 was confirmed by spectroscopic data: IR, 1H NMR, and ^{13}C NMR. The IR spectrum was characterized by the appearance of a sharp NH stretching peak at 3447 cm-1 instead of the forked peak corresponding to the NH2 group of compound 2 and the presence of two sharp peaks at 1320 and 1151 cm-1 for the SO2 functionality. The ^{1}H NMR spectrum showed a singlet upfield (CH_3) signal at δ 2.37 ppm and a typical AB system at δ 7.36 and 7.73 ppm attributed to the p-tolyl moiety. It also revealed an exchangeable singlet signal at δ 14.47 ppm corresponding to the sulfamoyl NH, which affirmed the condensation reaction. The ^{13}C NMR spectrum showed a characteristic signal at 21.49 ppm assigned for the methyl carbon. Fusion of 2 with ethyl acetoacetate afforded the methyl-substituted thiadiazolopyrimidinone (4). Cyclocondensation and formation of 4 were confirmed by the IR spectrum which showed the appearance of a C=O absorption band at 1694 cm-1 in addition to the disappearance of the NH2 forked band of its precursor. 1H NMR spectrum exhibited the appearance of a singlet signal at δ 2.31 ppm due to the CH3 protons and a singlet signal at δ 6.33 ppm corresponding to CH of pyrimidine. ^{13}C NMR spectrum showed the appearance of a signal at δ 163.55 ppm corresponding to the C=O group. It was expected that reaction of 2 with ethyl benzoylacetate under the same reaction condition will afford the phenyl substituted thiadiazolopyrimidinone derivative but the benzoyl acetamide derivative (5) was obtained instead, probably due to the steric effect of the phenyl ring which hindered cyclodehydration. The structure of 5 was confirmed by its ^{1}H NMR spectrum which showed the appearance of a singlet signal of the CH_2 protons at δ 4.41 ppm and signals of the phenyl protons in the range of δ 7.51-7.60 ppm in addition to the ^{13}C NMR spectrum which showed the appearance of signals at δ 166.66 and 194.36 ppm corresponding to the two C=O groups.

Scheme 1:



Reagents and conditions: (i) Ferric chloride, heat 80-90 °C, 45 min (ii) p-Tolylsulfonyl chloride, dry pyridine, reflux, 3h; (iii) Ethyl acetoacetate, fusion 12h; (iv) Ethyl benzoylacetate, fusion 12h.

3.2. Biological evaluation:

3.2.1. Cytotoxicity screening

The in vitro cytotoxicity of compounds 2-5 was evaluated against breast cancer (MCF-7) and human hepatoma (HepG2) cell lines by the MTT assay, using Staurosporine as a positive control. The results were expressed by median inhibitory concentration (IC50 in µM) as presented in Table 1.

Table 1
In vitro cytotoxic activities of compounds 2-5 and Staurosporine against MCF-7 and HepG2 cancer cell lines.

Compound	Cytotoxicity IC50 µM	
	MCF-7	HepG2
2	94.88±5.16	140.66±6.68
3	17.76±0.98	4.78±0.21
4	327.66±25.39	110.03±4.39
5	53.38±1.59	59.33±0.50
Staurosporine	17.81±0.66	25.53±1.56

The results showed that compounds 2, 4, and 5 exhibited weak or no cytotoxic activity against the tested cell lines with IC50 values in the high micromolar range (IC50 range =53.38 – 327.66 µM). Meanwhile, compound 3 displayed a potent growth inhibitory effect against MCF-7 and HepG2 cell lines with IC50 values of 17.76 and 4.78, respectively suggesting that elaborating 5-(4-chlorophenyl)-1,3,4-thiadiazole (2) with p-tolyl sulfonamide moiety in compound 3 is a beneficial strategy for increasing the antitumor activity of 1,3,4-thiadiazole derivatives. However, converting compound 2 into the bicyclic 5-methylthiadiazolopyrimidinone in 4 or attaching benzoyl acetamide moiety in 5 diminished or weakened the anticancer activity.

To confirm the enhancing effect of grafting a p-tolyl sulfonamide moiety on the cytotoxic activity of 5-(4-chlorophenyl)-1,3,4-thiadiazole and investigate the spectrum of activity, the cytotoxicity of the starting compound 2 and the most potent compound 3 were further evaluated against colon cancer (HCT116), prostate cancer (PC3) and lung cancer (A549) cell lines using the same assay method and Staurosporine as a positive control. The results were expressed by IC50 in µM as presented in Table 2.

The results revealed that the starting compound 2 showed weak cytotoxicity against the tested cell lines (IC_{50} range =24.64 – 58.19 µM). However, compound 3 exhibited remarkably high cytotoxicity against HCT116 and A549 cell lines with IC50 values of 2.32 and 4.40 µM, respectively. On the other hand, compound 3 was inactive against PC3 cells (IC_{50} = 139.59 µM). It is worth mentioning that, appending the chlorophenyl-1,3,4-thiadiazol scaffold in compound 2 with a p-tolyl sulfamoyl moiety in compound 3 resulted in a 5-fold, 35-fold, 25-fold, and a 10-fold increase in potency against MCF-7, HepG2, HCT116, and A459 cell lines, respectively. Furthermore, compound 3 exhibited higher activity against HCT116, HepG2 and A549 cells (IC50 = 2.32, 4.78 and 4.40 µM, respectively) than the positive control Staurosporine (IC50 = 30.05, 25.53 and 32.56 µM, respectively) and was equipotent to Staurosporine against MCF-7 cell line (IC50 values= 17.76 and 17.81 µM, respectively) (Tables 1 and 2). These results are consistent with our design hypnosis that the incorporation of substituted sulfonamide moiety may enhance the anticancer effect of the 2-amino-1,3,4-thiadiazole.

To ensure the selective cytotoxicity and safety of compound 3, its cytotoxicity was tested against a normal cell line (WI-38) and selectivity indices (SI) were calculated. Compound 3 revealed weak cytotoxic effect on normal cells (IC50 = 136.53 µM) with good to high selective cytotoxicity for MCF-7 (SI = 7.68), HepG2 (SI = 28.56), HCT116 (SI = 58.84) and A549 (SI = 31.02) cells over normal WI-38 cells (Figure 5), considering compound 3 as a potent and selective anticancer agent.

Table 2
In vitro cytotoxic activities of compounds 2 and 3 against HCT116, PC3, and A549 cancer cell lines and normal cells WI-38 using Staurosporine as a positive control

Compound	Cytotoxicity IC_{50} µM			
	HCT116	PC3	A549	WI-38
2	58.19±3.50	24.64±1.27	43.36±2.08	446.58±21.80
3	2.32±0.13	139.59±7.13	4.40±0.21	136.53±6.66
Staurosporine	30.05±2.61	20.85±0.68	32.56±3.66	76.29±3.72

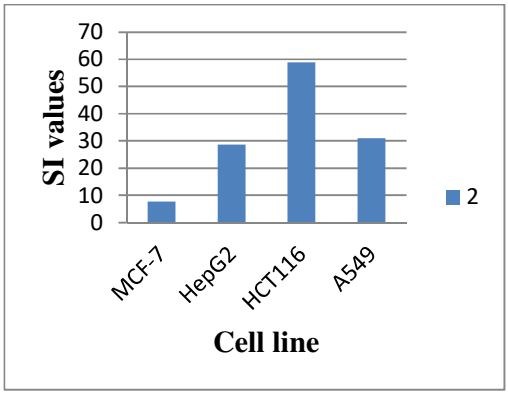


Figure 5: Chart illustrating SI values of compound 3 against MCF-7, HepG2, HCT116, and A549 cell lines.

$$SI = \frac{\text{IC50 against MCF-7 or HepG2 or HCT116 or A549 cells}}{\text{IC50 against } normal \text{ WI-38 cells}}$$

3.2.2. Carbonic anhydrase enzyme inhibition assay

The CA inhibition activity of synthesized compound 3 was screened using the stopped-flow assay method [21–28]. The inhibitory activity was tested against four isoforms of the enzyme, hCA I and II (cytosolic isoforms) and hCA IX and XII (transmembrane, tumor-associated isoforms). The standard drug used for this assay was acetazolamide (AZA), the well-known CA inhibitor. The results of the assay are displayed in Table 3. The results revealed that the tumor-associated isoforms hCA IX and XII (Ki = 1.00 and 2.60 µM) were more sensitive to the tested compound than the cytosolic isoforms hCA I and II (Ki = 86.20 and 30.90 µM), implying that the anticancer activity of compound 3 is a least partly attributed to inhibition of the tumor-associated CA isoforms IX and XII.

High inhibitory activity against tumor-associated carbonic anhydrase isoforms hCA IX and XII is correlated to good anticancer activity, while inhibition of the off-target cytosolic isoforms hCA I and II will result in side effects [13]. In order to examine the differential inhibitory activity of compound 3 toward the tested isoforms, the selectivity indices (SI) of hCA II/IX and hCA II/XII, were calculated (Table 3). The higher the inhibitory activity against hCA IX and hCA XII isoforms over hCA II isoform and hence the higher the SI, the more effective and safe a compound would be. In this regard, compound 3 exhibited remarkable selectivity towards hCA IX and XII over hCA II (SI = 30.90 and 11.88, respectively) much higher than that of AZA (SI = 0.16 and 2.10, respectively).

3.2.3. Radiolabeling and in vivo bio-distribution of radio-iodinated compound 3

The radiopharmaceutical chemistry field represents an intensive approach for studying ADME parameters of newly synthesized molecules. Radioactive iodine is very efficient for radio-iodination of organic compounds to act as a screening probe of their in-vivo bio-distribution pattern as radioiodine is effectively compatible with a wide scope of organic compounds besides its easy physical radio-imaging screening [29]. Since compound 3 exhibited potent and selective anticancer activity towards HCT116, HepG2, and A549 cells, its in-vivo bio-distribution pattern was investigated after radiolabeling with iodine.

3.2.3.1. Radiosynthesis of radio-iodinated compound 3:

The highest radiosynthesis yield of radio-iodinated compound 3 was 95.16±1.28%. Such maximum yield was obtained using 300 µg chloramine-T, pH 4, 6 µg of compound 3, and 20 min reaction time (Figure 6).

3.2.3.2. Radioactive in-vivo tracing studies of radio-iodinated compound 3 in normal mice model:

Figure 7 showed the biodistribution pattern of radio-iodinated compound 3 in the normal mice model. Figure 7 (a) presented % ID/g organ for excretory organs kidneys, liver, and intestine and clarified that radio-iodinated compound 3 is mainly excreted via renal and hepatobiliary pathways. Figure 7 (b) illustrated the %ID/g organ of all non-target organs for radioiodinated compound 3, it showed a normal blood clearance pattern and clarified that there is no significant accumulation of the radio-iodinated-3 compound in most non-target organs (spleen, heart, brain, and muscle). The data also show a decrease in the blood concentration of 3 with time and intensive accumulation in target organs [gastrointestinal tract (GIT) and lungs] indicating the effective transfer of compound 3 to the target organs. These results along with the high in vitro cytotoxic effect of 3 toward colon HCT116 and lung A549 cell lines suggest that compound 3 may act as an effective and selective anticancer agent against colon and lung cancer with minimal effect on non-target organs.

Table 3
The inhibitory activity of compound 3 against human CA isoforms hCA I, II, IX, and XII and its selectivity for the inhibition of hCA IX and XII over hCA II, using AZA as a standard drug

Name	K_i (µM)[a]				SI[b]	
	hCA I	hCA II	hCA IX	hCA XII	hCA II/IX	hCA II/XII
3	86.20	30.90	1.00	2.60	30.90	11.88
AZA	0.25	0.012	0.075	0.0057	0.16	2.10

a.
Mean from 3 different assays, by a stopped-flow technique (errors were in the range of □ 5-10 % of the reported values).
b.
SI (selectivity index) is a ratio between the Ki values observed for two hCA isoforms (a low-value index is indicative of weak selectivity).

$$SI = \frac{\text{Ki value of hCA II}}{\text{ki value of hCA IX or hCA XII}}$$

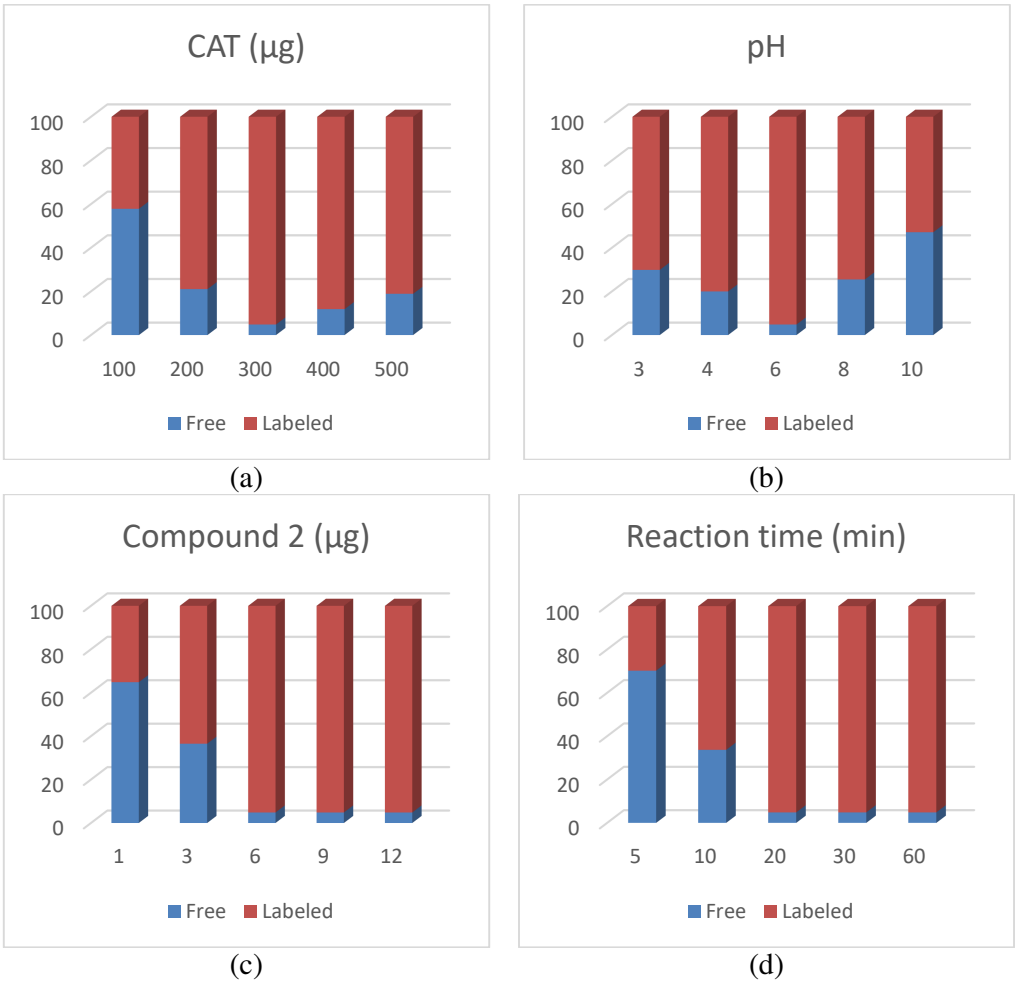
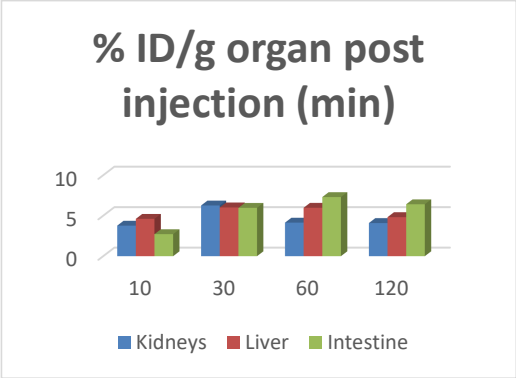
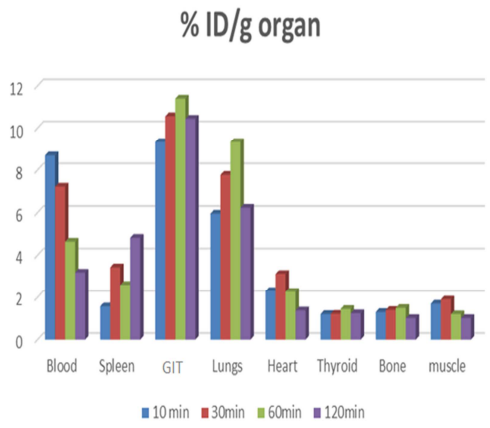


Figure 6: Variation of radiosynthesis yield of radio-iodinated compound 3 concerning (a) chloramine-T amount, (b) pH, (c) compound 3 amount, and (d) reaction time.



(a)



(b)

Figure 7: Percent injected dose per gram organ of radio-iodinated compound 3 in normal mice model in (a) excretory organs and (b) non-target organs

4. Conclusion:

In this work, a new 1,3,4-thiadiazole-carrying substituted benzenesulfonamide moiety was designed and synthesized as a selective cytotoxic agent. The novel compound 3 was characterized by spectral and micro-analytical analyses. Compound 3 was assessed for cytotoxic activity against five different cancer cell lines and a normal cell line where it showed high potency and selectivity against colorectal (HCT-116), lung (A549), and liver (HepG2) cancer cell lines. Furthermore, carbonic anhydrase inhibitory activity assay results revealed that compound 3 has high selectivity against tumour-associated CA isoforms IX and XII over cytosolic isoform II. Finally, a pharmacokinetic study of the 125I radio-iodinated compound 3 was performed and bio-distribution results showed intensive accumulation in the lung and gastrointestinal tract, proving that compound 3 can be considered an anticancer agent targeting lung and gastrointestinal cancers.

5. Conflict of interest:

All authors declared no conflict of interest.

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