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Synthesis of New Sulfonamide Derivatives-Phenylalanine and Proline Ester Conjugate Using Succinamide Spacer as Anticancer Agents

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ABSTRACT

Objective: *New compounds were designed and synthesized using sulfonamide derivatives of cytosine and 9-methyl adenine that conjugated with amino acids ester through succinamide spacer, and suspected to have anticancer activity.*

Methods: *4-acetamidobenzenesulfonyl chloride was reacted with cytosine and 9- methyl adenine to form compounds (Ib and Id) respectively as sulfonamide derivatives, that were deacetylated to obtain compounds (Ic and Ie) respectively. Esterification of amino acids was done to obtain phenyl alanine methyl ester HCl (IIa) and proline methyl ester HCl (IIb), that reacted with succinic anhydride to form compounds IIIa and IVa respectively as carboxylic acid derivatives. Each one of the compounds (IIIa and IVa) was reacted with compound Ic through formation of amide bond and leads to the formation of the targeting compounds (A and D) respectively.*
The compounds (IIIa and IVa) were also reacted with compound Ie lead to the formation of the targeting compounds (G and J) respectively. The study has also employed an in vitro evaluation of the cytotoxic activity of the synthesized compounds (G and J) on two cancer cell lines: the murine mammary adenocarcinoma cell line (AMN3), the human breast cancer cell line(AMJ13), and one normal cell line: the rat embryo fibroblast cell line (Ref), at different concentrations of the tested compounds and different treatment exposure time (48 hrs. and 72 hrs.).
The titled compounds were characterized and identified by elemental microanalysis, I.R spectra and ^{1}HNMR study, and it was found that all the results showed good agreements with the proposed chemical structures of the synthesized compounds.

Results: *Cytotoxicity study of compounds G and J indicate that both compounds have inhibitory effect on the murine mammary adenocarcinoma cell line (AMN3), and the human breast cancer cell line (AMJ13) depending on the concentration used of the tested compounds, but no significant effect on the rat embryo fibroblast cell line (Ref, the normal cell line).*

Conclusion: *The results of this study indicate that the new designed compounds were successfully achieved and they have selectivity in their action toward inhibition of cancer cells.*

Key words: *sulfonamide derivative, succinamide derivative, cytotoxicity*

INTRODUCTION

Sulfonamides are one of the drugs that have been in clinical use for over 70 years. They play an important role in medicinal chemistry: many antimicrobial, anti-inflammatory, antipsychotic, antihypertensive, hypoglycemic, and anticancer drugs contain the sulfonamide subunit [1]. New sulfonamides have shown good anticancer activity against breast cancer cells [2, 3]. A new set of sulfonamide derivatives of potential antitumor activity were designed and synthesized as histone deacetylase inhibitors (HDACIs) [4]. Anthranilate sulfonamides have been recently reported to be cytotoxic acting as methionine aminopeptidase- 2 (MetAP-2) inhibitors [5]. The conjugation of hydroxamic and benzene sulfonamide moieties in the same molecular entity, for dual inhibition of MMPs (Matrix metalloproteinases) and CAs (Carbonic anhydrases), has been recently reported [6] and its potential pharmacologic interest has been outlined, since some members of MMP family are also known to be involved in carcinogenesis and tumor progression processes [7]. CA IX and CA XII have been shown to be associated with tumors [8], the potential use of aromatic/ heterocyclic sulfonamides as CA inhibitors has been little explored to date in the treatment of cancer [9].

Nucleoside-derived drugs are very important in the treatment of several cancers [10]. The pyrimidine and their bioisosteres, are heterocyclic compounds with potent biological functions including anticancer [11]. Purine is a heterocyclic, aromatic, organic compound, consisting of a pyrimidine ring fused to an imidazole ring [12, 13], purine analogs are widely used against various diseases, particularly cancer such as the use of 6-mercaptopurine [14] and thioguanine in cancer treatment [15]. The system large amino acid transporter (LAT) plays a critical role in the absorption of amino acids from the small intestine, as well as in movement of amino acids across the blood–brain barrier, the placenta, and the proximal tubules of the kidneys [16]. Among the known LATs, LAT1 has garnered particular attention because of its limited distribution and higher expression in malignant tumors. So, it could be used as target in chemotherapy [17].

MATERIALS AND METHODS

All chemicals were reagent grade and obtained from standard commercial sources. Acetanilide (Riedel-Dehaen, Germany), Chlorosulfonic acid (Alpha Chemica, India), Cytosine (BDH, England), 9-Methyl adenine (Hangzhou hyper chemicals limited, China), L-phenyl alanine (SCR, China), L-proline (Chemical point, Germany), Succinic anhydride (Himedia, India). Melting points of the compounds and their intermediates were determined by capillary tube method on Stuart (U.K) Electrical melting point apparatus, ascending thin layer chromatography (TLC) was run on silica gel GF254 (type 60) pre-coated aluminum sheets, Merck (Germany) to check the purity and the reactions progress. The products were detected by reacting with iodine vapor or by irradiation with UV light. Infrared spectra were recorded on F.T. IR Spectrophotometer Shimadzu (Japan), CHNS microanalysis was done using Elemental micro-analyzer Vario Micro (Germany), and the 1HNMR spectra were recorded on (Bruker, Germany NMR Spectrometer 400 MHz, Advance III 400 spectrometer) with TMS as an internal standard.

Chemical synthesis

Synthesis of compound 4-acetamidobenzenesulfonyl chloride (Ia) [18]:

Acetanilide (2.5gm, 18.49 mole) was put in dry conical flask and melted on a direct flame. The melted acetanilide was distribute in a thin layer, round and over the bottom of the flask, cooled the flask to let the acetanilide solidify again, with cooling in ice bath Chlorosulfonic acid (6ml, 94 mmole) was added and immediately connect the flask to the gas trap. The flask was swirled after removing it from ice bath for 15 min. The mixture was heated on a water bath for 20 minutes to complete the reaction, the flask was cooled under the tap water and the mixture was poured in a thin stream into 75ml of crushed ice water mixture in a beaker with stirring. The precipitated p-acetamidobenzenesulfonyl chloride in the form of granular white solid, was stirred, filtered off at the pump and washed it with a little cold water, pressed and drained well. The crude product was washed with toluene and recrystallized from chloroform. White to pale yellow solid, yield 85%, melting point 142-145 °C.

General procedure for the synthesis of N-substituted 4-acetamidobenzenesulfonamides (Ib and Id) [19]:

4-acetamidobenzene sulfonyl chloride (6.7 mmol, 1.56gm) was added gradually to a suspension of related amine (6.7 mmol) and pyridine (6.7 mmol, 0.54 ml) in DMF (25 ml), with stirring at room temperature for 15 min. Then the mixture was heated for 12 hrs, the solvent was evaporated to dryness under vacuum. The crude material was washed with chloroform, diethyl ether, and recrystallized from ethanol.

N-(4-(N-(2-oxo-1, 2-dihydropyrimidin-4-yl) sulfamoyl) phenyl) acetamide), compound Ib:

Off white powder, yield 82%, melting point 242-245°C. IR (υ= cm-1, KBr): 3331(NH) of 2o amide; 3190 (NH) of sulfonamide; 3057 (CH) aromatic; 2924 and 2885(CH) asymmetric and symmetric respectively of CH3; 1703 (C=O) aniline; 1660(C=O) of amide of cytosine; 1593, 1527, 1492 (C=C) aromatic; 1315, 1170 (O=S=O) asymmetric and symmetric respectively.

(N-(4-(N-(9-methyl-9H-purin-6-yl) sulfamoyl) phenyl) acetamide), compound Id:

Off white powder, yield 56%, melting point 218-220°C. IR (υ= cm-1, KBr): 3298(NH) of anilide; 3182 (NH) of sulfonamide; 3105 (CH) aromatic; 3000 and 2794(CH) asymmetric and symmetric respectively of CH3; 1700 (C=O) anilide; 1597, 1535, 1496 (C=C) aromatic; 1319, 1172 (O=S=O) asymmetric and symmetric respectively.

General procedure for the synthesis of N-substituted 4-aminobenzenesulfonamides, compounds Ic and Ie [19]:

HCl (6N, 10 ml) was added to related N-substituted 4-acetamidobenzenesulfonamides (Ib, Id) (1gm) and refluxed for 4 hrs. After this time, the product was neutralized by 25% sodium hydroxide; the obtained product was filtered and washed with ethanol to give compound Ic and Ie respectively.

(Amino-N-(2-oxo-1, 2-dihydropyrimidin-4-yl) benzene sulfonamide), Compound Ic:

Pale yellow powder, yield 64%, melting point 215-218°C. IR (υ= cm-1, KBr): 3483 and 3385(NH) asymmetric and symmetric stretch respectively of 1o amine; 3331 (NH) of 2o amide; 3174(NH) of sulfonamide; 3066 (CH) aromatic; 1664 (C=O) 2o amide (cytosine); 1602, 1581, 1502 (C=C) aromatic; 1363, 1180 (O=S=O) asymmetric and symmetric respectively.

(4-Amino-N-(9-methyl-9H-purin-6-yl) benzene sulfonamide), Compound Ie:

Pale brown powder, yield 60%, melting point 198-200°C (decomposed). IR (υ= cm-1, KBr): 3489 and 3383(NH) asymmetric and symmetric stretch respectively of 1o amine; 3238(NH) of sulfonamide; 3103 (CH) aromatic; 2922 and 2870 (CH) asymmetric and symmetric stretch respectively of CH3; 1600, 1575, 1502 (C=C) aromatic; 1383, 1175(O=S=O) asymmetric and symmetric respectively.

General procedure for the synthesis of amino acid methyl ester hydrochloride (IIa and IIb): [20]

A suspension of related amino acid (30.26 mmol) in absolute methanol (75 ml) was cooled down to -10 ºC and thionyl chloride (36.32 mmol, 2.6 ml) was added drop wise, the reaction mixture was stirred at 40 ºC for 3 h, then refluxed for 3 h, and left at room temperature overnight, the solvent was evaporated to dryness under vacuum, redissolved in methanol and evaporated, this process was repeated several times. The resulting solid product was collected and dried under vacuum, to give crude methyl ester hydrochloride. The crude material was dissolved in minimum amount of hot methanol. Slow addition of excess of diethyl ether followed by cooling to 0 °C gave pure crystals. The crystals were collected on the following day and washed twice with diethyl ether: methanol (5:1) mixture and dried under vacuum to get pure compound.

L-phenylalanine methyl ester HCl (IIa):

White crystals, yield 90%, melting point 158-160°C. IR (υ= cm-1, KBr): 3003-2800(NH) stretch of ammonium include (CH) aromatic and CH3; 1741(C=O) ester; 1627(NH) bending of ammonium; 1583 and 1496 (C=C) aromatic.

L-proline methyl ester HCl (IIb):

Oily, yield 98%, IR (υ= cm-1, KBr): 3425(NH) stretch of ammonium salt of 2o amine; 2955 and 2740 (CH) of CH3 and CH2; 1743(C=O) ester; 1627(NH) bending of amine salt.

Synthesis of compound 4-(1-methoxy-1-oxo-3-phenylpropan-2-ylamino)-4-oxobutanoic acid (IIIa) [21]:

To a suspension of IIa (4.6 mmol, 1 gm) in chloroform (25 ml), N-methyl morpholine (4.6 mmol, 0.5ml) was added drop wise, and stirring at room temperature until clear solution is predominant, then succinic anhydride (4.6 mmol, 0.46 gm) was added gradually to the reaction mixture, the reaction mixture was stirred at room temperature for about 2 hours. The organic layer was washed with distilled water (2 ×20ml), dried with anhydrous magnesium sulphate and filtered; the chloroform was evaporated to yield an oily residue. Yield 78%. IR (υ= cm-1, KBr): 3306(NH) stretch of 2o amide and (OH) stretch of carboxylic acid; 3030 (CH) stretch of aromatic; 2953 (CH) asymmetric stretch of CH3; 2933 (CH) asymmetric stretch Of CH2; 1732(C=O) ester; 1716 (C=O) carboxylic acid; 1647 (C=O) 2o amide; 1531 (C=C) stretch of aromatic.

Synthesis of 4-(2-(methoxycarbonyl) pyrrolidin-1-yl)-4-oxobutanoic acid (IVa) [21]:

To a suspension of IIb (6 mmol, 1 gm) in ethyl acetate (25 ml), N-methyl morpholine (6 mmol, 0.66ml) was added drop wise, and stirring at room temperature, then succinic anhydride (6 mmol, 0.6 gm) was added gradually to the reaction mixture, the reaction mixture was stirred at room temperature for about 2 hours. N-methyl morpholine hydrochloride was filtered off and the filtrate was collected, ethyl acetate was evaporated to yield an oily residue. Yield 92%. IR (υ= cm-1, KBr): 3481(OH) stretch of carboxylic acid; 2955, 2933 and 2881 (CH) asymmetric and symmetric stretch of CH3 and CH2; 1732(C=O) ester and carboxylic acid; 1639 (C=O) 3o amide.

General procedure for the synthesis of compounds A, D, G and J: [22]

Thionyl chloride (0.82 mmole, 0.06ml) was added drop wise to a stirred solution of related carboxylic acid derivatives IIIa, IVa (0.82mmole) in dry chloroform (25ml) at −5 ºC, and the reaction mixture was refluxed for 3 hrs. Chloroform was evaporated and the residue was redissolved in chloroform, and evaporated under vacuum. An oily residue of acid chloride was obtained and dissolved in dry THF (10 ml) and added drop wise to a stirred solution of related N-substituted 4-aminobenzenesulfonamides Ic, Ie (0.82mmole) and N-methyl morpholine (0.82mmole, 0.09ml) in DMF (10ml). The reaction mixture was stirred over night at room temperature. The solvent was evaporated and the residue was washed with chloroform, triturated with diethyl ether, and recrystallized from ethanol.

Compound A, (methyl (4-oxo-4-((4-(N-(2-oxo-1, 2-dihydropyrimidin-4-yl) sulfamoyl) phenyl) amino) butanoyl) phenylalaninate):

Yellowish orange powder, yield 68%, melting point 75-78°C, IR (υ= cm-1, KBr): 3327 (NH) of 2o amide; 3213(NH) of sulfonamide; 3034 (CH) aromatic; 2947, 2931 and 2885 (CH) asymmetric and symmetric stretch of CH3 and CH2; 1728 (C=O) ester; 1658 (C=O) 2o amide; 1600, 1514 (C=C) aromatic; 1321, 1180 (O=S=O)asymmetric and symmetric.1HNMR(400MHz), DMSO-d6 , δ, ppm): 2.9(d, 2H, -CH2-Ar), 3.6(s, 3H, CH3-O), 3.9(s, 4H, -CH2-CH2-C=O), 4.2(t, 1H, -CH-), 6.2-6.8(m, 2H, pyrimidin-one), 7-8(m, 9H, Ar-), 11.5(s, 1H, -NH-C=O), 11.9(s, 1H,- NH-Ar), 12.3(s, 1H, -NH-pyrimidin), 12.9(s, 1H, -NH-S). CHNS Calculated forC24H25N5O7S: C, 54.64; H, 4.78; N, 13.28; S, 6.08. Found: C, 54.48; H, 4.813; N, 13.16; S, 6.45.

Compound D, Methyl (4-oxo-4-((4-(N-(2-oxo-1, 2-dihydropyrimidin-4-yl) sulfamoyl) phenyl) amino) butanoyl) prolinate:

Yellowish green powder, yield 72%, melting point 67-68°C, IR (υ= cm-1, KBr): 3385 (NH) of 2o amide; 3182(NH) of sulfonamide; 3062 (CH) aromatic; 2949 and 2877 (CH) asymmetric and symmetric stretch Of CH3 and CH2; 1726 (C=O) ester; 1641 (C=O) amide; 1600, 1512 (C=C) aromatic; 1328, 1178(O=S=O)asymmetric and symmetric respectively.1HNMR(400MHz), DMSO-d6 , δ ppm): 2(m, 2H, CH2-cyclo-), 2.2(m, 2H, CH2-cyclo-), 3.5(s, 4H, -CH2-CH2-C=O), 3.66(s, 3H, CH3O), 3.8(t, 2H, CH2-cyclo-), 4.1(t, 1H, -CH-), 6.7-6.8(m, 2H, pyrimidin-one), 7-8(m, 4H, Ar-), 11.6(s, 1H, -NH-C=O), 12.1(s, 1H, -NH-pyrimidin-), 12.9(s, 1H, -NH-S-). CHNS Calculated forC20H23N5O7S: C, 50.31; H, 4.86; N, 14.67; S, 6.72. Found: C, 49.85; H, 4.95; N, 14.87; S, 6.42.

Compound G, (Methyl (4-((4-(N-(9-methyl-9H-purin-6-yl) sulfamoyl) phenyl) amino)-4-oxobutanoyl) phenylalaninate):

Yellow powder, yield 66%, melting point 72-73°C, IR (υ= cm-1, KBr): 3338 (NH) of 2o amide; 3257(NH) of sulfonamide; 3020 (CH) aromatic; 2943 and 2864 (CH) asymmetric and symmetric stretch Of CH3 and CH2; 1728 (C=O) ester;1685 and 1660 (C=O) amide;1599, 1516, 1498 (C=C) aromatic; 1323, 1165(O=S=O)asymmetric and symmetric respectively.1HNMR(400MHz), DMSO-d6 , δ ppm): 2.98(d, 2H, CH2-Ar), 3.4(s, 4H, -CH2-CH2-C=O), 3.7(s, 3H, CH3-N),3.8(s,3H,CH3-O), 4.8(t, 1H, -CH-), 5.8(s, 1H, purine), 6.2(s, 1H, purine), 7-8(m, 9H, Ar-), 12.2(s, 1H, -NH-C=O), 12.5(s, 1H, -NH-Ar), 12.7(s, 1H, -NH-S). CHNS Calculated forC26H27N7O6S: C, 55.21; H, 4.81; N, 17.33; S, 5.67. Found: C, 54.66; H, 4.88; N, 17.86; S, 5.34.

Compound J, (Methyl (4-((4-(N-(9-methyl-9H-purin-6-yl) sulfamoyl) phenyl) amino)-4-oxobutanoyl) prolinate):

Mustard powder, yield 67%, melting point 60-62°C, IR (υ= cm-1, KBr):3327 (NH) of 2o amide; 3248(NH) of sulfonamide; 3080 (CH) aromatic; 2953 and 2929 and 2881 (CH) asymmetric and symmetric stretch of CH3 and CH2; 1728 (C=O) ester; 1660 and 1631 (C=O) amide;1600, 1510 (C=C) aromatic; 1325, 1180(O=S=O)asymmetric and symmetric respectively.1HNMR(400MHz), DMSO-d6 , δ ppm): 2.1(m, 2H, CH2-cyclic), 2.3(m, 2H, CH2-cyclic), 3.1(s, 4H, CH2-CH2-C=O), 3.5(s, 3H, CH3-N), 3.7(s, 3H, CH3-O), 4(t, 2H, -CH2-N), 4.65(t, 1H, -CH-), 5.7(s, 1H, purine), 6.5(s, 1H, purine), 7.5-8(m, 4H, Ar-), 12.4(s, 1H, -NH-C=O), 12.81(s, 1H, -NH-S). CHNS Calculated forC22H25N7O6S: C, 51.25; H, 4.89; N, 19.02; S, 6.22. Found: C, 50.76; H, 4.95; N, 19.45; S, 6.70.

Cytotoxic Activity Study

The evaluation of cytotoxic activity was done at the Iraqi Centre for Cancer and Medical Genetic Research (ICCMGR) using two types of tumor cell lines: murine mammary adenocarcinoma cell line (AMN3) [23] and primary tumour of a 70 years old Iraqi woman with a histological diagnosis of infiltrating ductal carcinoma (breast cancer cell line AMJ13) [24] and one type of normal cell line: rat embryo fibroblast cell line(Ref) [25]. They were maintained in growth medium supplemented with 10% fetal calf serum and seeded on micro-titration (96- well plates at a concentration of 1×104 cells/well), and various concentrations of tested compounds (G and J) were added from (3.125 to 100 µg/ml) prepared by serial twofold dilutions using maintenance media from stock solution of test sample in triplicate form of each concentration. The negative control wells contained only the cells with culture media, then the 96-well cell culture plate incubated at 37°C in an incubator supplemented with 5% CO2 for 2 different times (48, 72) hrs [26]. The cytotoxic activity of compounds was evaluated by Crystal violet assay, the optical density of each well was measured by using ELISA (Enzyme Linked Immuno Sorbent Assay) reader at a transmitting wave length on 492 nm. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as (A-B)/A ×100, where A is the mean optical density of untreated wells (control), and B is the optical density of treated wells [27]. Data were analyzed by 2-way analysis of variance with ANOVA. The level of significance ($p<0.05$) was used for analysis of the results.

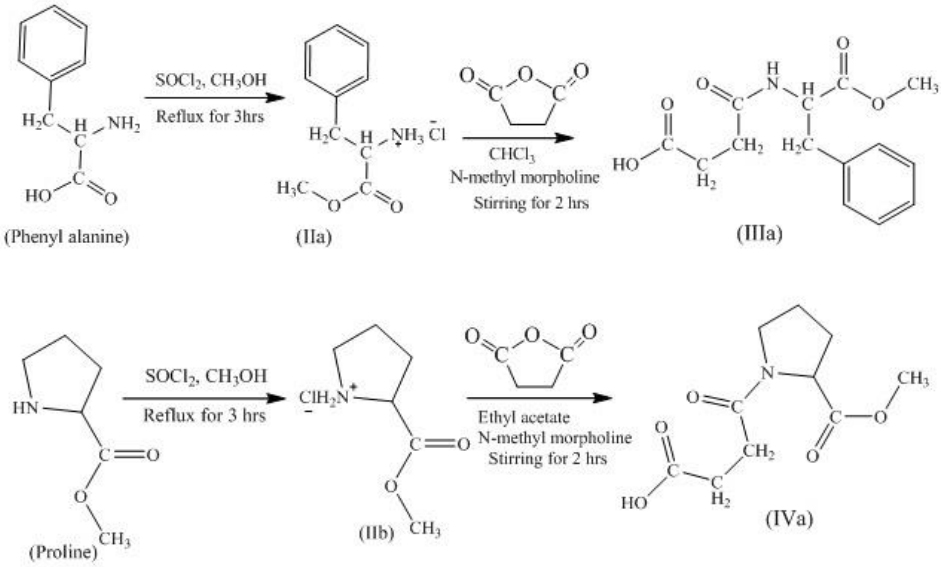
RESULTS AND DISCUSSION

Chemistry

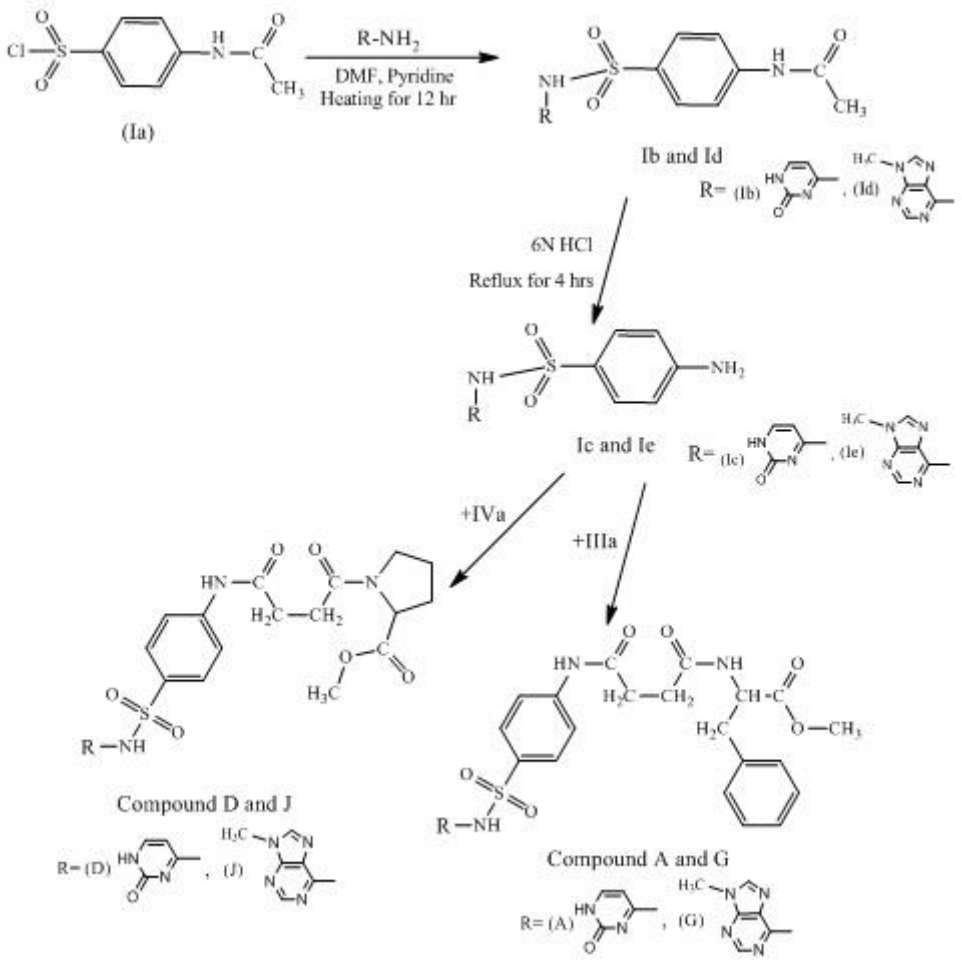
The synthesis of the compounds (A, D, G and J) was accomplished and outlined in the schemes (1and 2), which illustrated the reactions sequences for the all synthesized compounds. Reaction of acetanilide with Chlorosulfonic acid lead to the formation of 4-acetamidobenzenesulfonyl chloride(Ia), which was reacted with cytosine and 9- methyl adenine, to form compounds (Ib and Id)

respectively, that were deacetylated by heating with 6N HCl to obtain compounds (Ic and Ie) respectively. Esterification of amino acids was done by activation of the amino acid by thionyl chloride to get acyl chloride that attacks methanol producing methyl ester of the selected amino acid. Compounds IIa and IIb were synthesized by this method. Reaction of compound IIa with succinic anhydride afforded compounds IIIa. Reaction of compound IIb with succinic anhydride afforded compounds Iva.

Each one of the compounds (IIIa and IVa) was reacted with thionyl chloride in dry chloroform and refluxed for 3 hours to form acyl chloride that was reacted with compound Ic leads to the formation of the targeting compounds (A, D) respectively. The acyl chloride of the compounds (IIIa and IVa) reacted with compound Ie leads to the formation of the targeting compounds (G, J) respectively.



Scheme-1: Synthesis of compounds IIIa and IVa



Scheme-2: Synthesis of compounds A, D, G and J

Cytotoxicity study

The cytotoxic activities (cell viability assay) of compounds (G, J) were evaluated by Crystal violet assay [28] (viable cells will uptake the dye and the dead cells will not). Three cell lines were studied (AMN-3 passage no.117, AMJ-13 passage no. 50, Ref cell lines passage no. 120) at two times of exposure (48, 72 hours).

Table-1: The effects of different concentrations of compound G on growth of AMN3, AMJ13, and Ref cell lines

	48 hours			**72 hours**		
Conc. µg/ml	**AMN3**	**AMJ13**	**Ref**	**AMN3**	**AMJ13**	**Ref**
3.125	52.0 (6.4)	49.6 (1.6)	0.0 (4.4)	52.7 (0.2)	21.3 (2.8)	2.8 (2.8)
6.25	28.1 (1.0)	25.2 (0.8)	-1.3 (1.3)	22.2 (0.2)	0.0 (0.8)	-2.8 (2.8)
12.5	25.1 (0.6)	4.9 (1.4)	2.6 (2.6)	19.6 (7.8)	-0.5 (0.9)	2.8 (2.8)
25	8.2 (0.6)	8.1 (2.2)	2.6 (2.6)	19.6 (5.2)	2.3 (2.0)	6.4 (1.9)
50	23.4 (7.9)	5.7 (2.9)	-2.6 (5.1)	23.5 (5.9)	0.0 (0.8)	-2.8 (1.4)

100	26.9(10.5)	4.9 (3.7)	2.6 (5.1)	35.3(11.8)	1.4 (2.4)	0.0 (5)

Control= 0% inhibition; Positive results of the % growth inhibition indicate anti-proliferation; Negative results of the % growth inhibition indicate proliferation

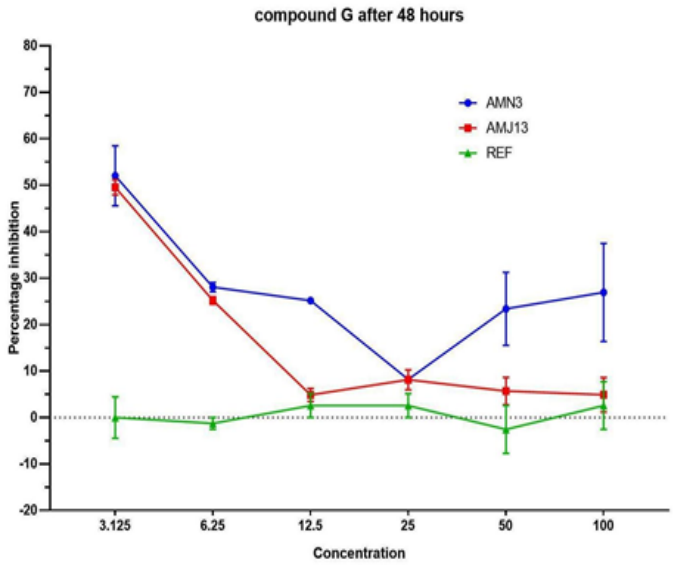


Figure-1: Inhibition rate (%IR) of different concentrations of compound G on three cell lines at 48 hrs.

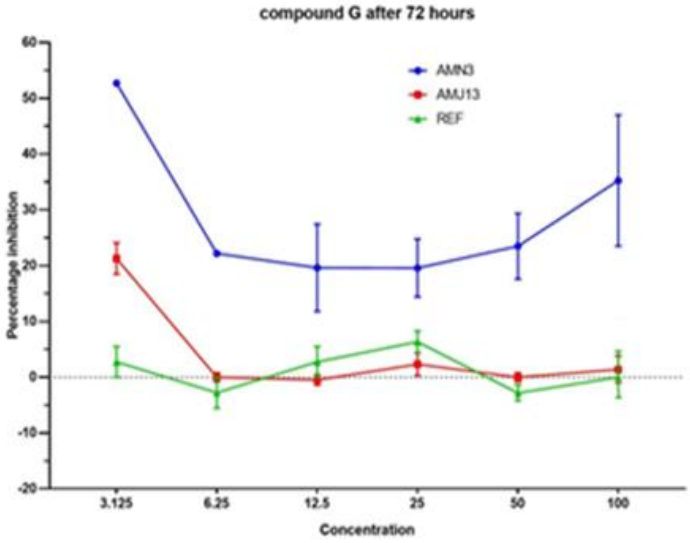


Figure-2: Inhibition rate (%IR) of different concentrations of compound G on three cell lines at 72hrs.

Data were analyzed by 2-way analysis of variance with ANOVA. The level of significance ($p<0.05$) was used for analysis of the results

Table (2): post-hoc test of each pair of concentrations for compound G (columns comparison) showing their p values

	48 hours			72 hours		
Concμg/ml	AMN3	AMJ13	Ref	AMN3	AMJ13	Ref
3.125 vs. 6.25	0.0047	0.0039	NS	0.0008	0.0329	NS
6.25 vs. 12.5	NS	0.0229	NS	NS	NS	NS
12.5 vs. 25	NS	NS	NS	NS	NS	NS
25 vs. 50	NS	NS	NS	NS	NS	NS
50 vs. 100	NS	NS	NS	NS	NS	NS
NS: non-significant						

Table-3: post-hoc test of each pair of tissue line at fixed concentration of compound G (row comparison) showing their p values

	48 hours			72 hours		
Conc. μg/ml	AMN3 vs. Ref	AMJ13 vs. Ref	AMN3 vs. AMJ13	AMN3 vs. Ref	AMJ13 vs. Ref	AMN3 vs. AMJ13
3.125	<0.0001	<0.0001	NS	<0.0001	0.0234	0.0001
6.25	<0.0001	<0.0001	NS	0.0019	NS	0.0058
12.5	0.0020	NS	0.0057	0.0421	NS	0.0131
25	NS	NS	NS	NS	NS	0.0131
50	0.0004	NS	0.0171	0.0011	NS	0.0011
100	0.0009	NS	0.0026	<0.0001	NS	<0.0001
NS: non-significant						

The results indicate that significant inhibitory effects appeared in the (AMN3) murine mammary adenocarcinoma cell line more than human breast cancer (AMJ13) cell line, compound G act at low concentrations in both cancer cell lines, while no significant inhibitory effect of the compound appear in normal cell line (Ref).

Table-4: The effects of different concentrations of compound J on growth of AMN3, AMJ13, and Ref cell lines

	48 hours			**72 hours**		
Conc. μg/ml	**AMN3**	**AMJ13**	**Ref**	**AMN3**	**AMJ13**	**Ref**
3.125	21.6 (6.9)	18.7 (8.0)	0.0 (0.0)	11.8 (3.4)	1.4 (0.8)	4.2 (2.4)
6.25	24.0 (3.8)	3.3 (2.2)	0.3 (1.6)	9.6 (0.4)	-0.5 (0.5)	0.0 (4.8)
12.5	17.0 (1.5)	5.7 (6.3)	1.5 (2.4)	17.1 (6.2)	0.0 (0.8)	0.0 (4.8)
25	42.7 (2.5)	32.5 (3.5)	0.0 (4.4)	46.1 (1.0)	51.4 (3.5)	-2.8 (5.6)
50	45.6 (1.0)	43.1 (3.5)	-2.6 (2.6)	48.0 (1.0)	51.4 (4.0)	2.8 (5.6)

100	55.6 (2.1)	51.2 (2.8)	0.0 (7.7)	63.7 (1.5)	61.6 (2.0)	1.4 (3.5)

Control= 0% inhibition; Positive results of the % growth inhibition indicate anti-proliferation; Negative results of the % growth inhibition indicate proliferation

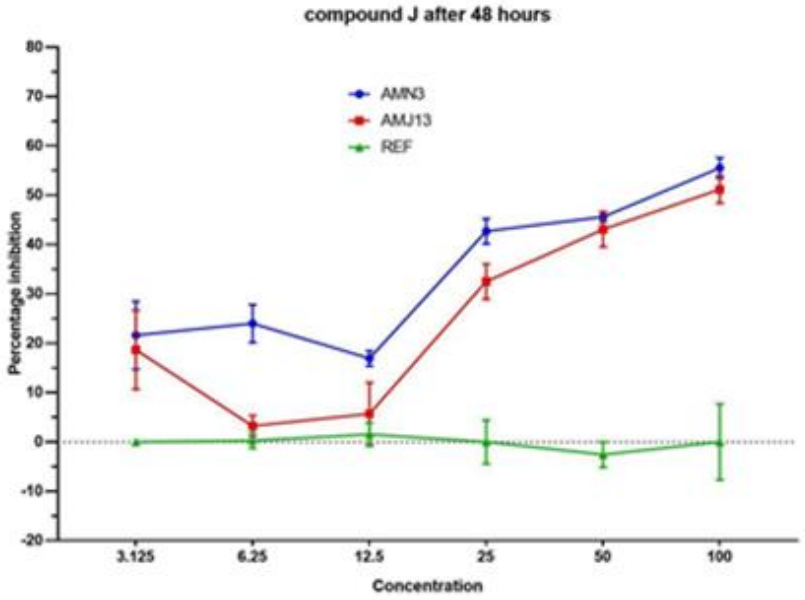


Figure-3: Inhibition rate (%IR) of different concentrations of compound J on three cell lines at 48 hrs.

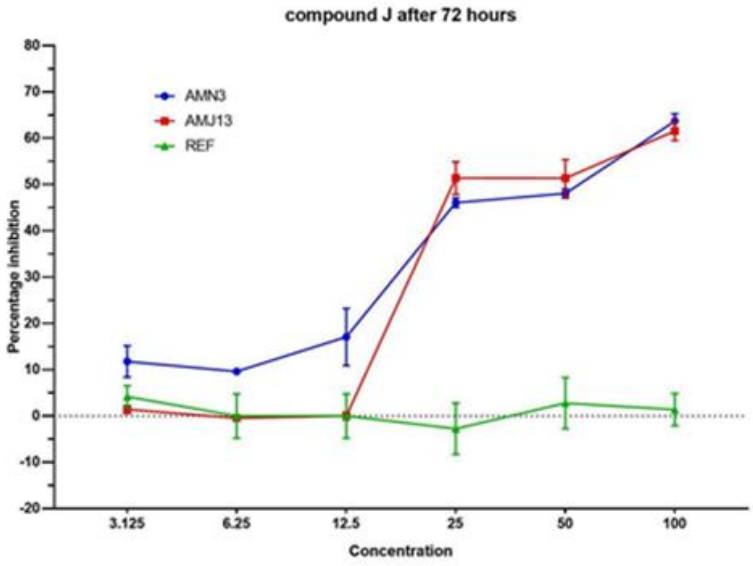


Figure-4: Inhibition rate (%IR) of different concentrations of compound J on three cell lines at 72 hrs.

Data were analyzed by 2-way analysis of variance with ANOVA. The level of significance ($p<0.05$) was used for analysis of the results

Table -5: post-hoc test of each pair of concentrations of compound J (columns comparison) showing their p values

	48 hours	72 hours

Conc.µg/ml	AMN3	AMJ13	Ref	AMN3	AMJ13	Ref
3.125 vs. 6.25	NS	NS	NS	NS	NS	NS
6.25 vs. 12.5	NS	NS	NS	NS	NS	NS
12.5 vs. 25	0.0013	0.0008	NS	<0.0001	<0.0001	NS
25 vs. 50	NS	NS	NS	NS	NS	NS
50 vs. 100	NS	NS	NS	0.0302	NS	NS
NS: non-significant						

Table-6: post-hoc test of each pair of tissue line at fixed concentration for compound J (row comparison) showing their p values

	48 hours			72 hours		
Conc. µg/ml	AMN3 vs. Ref	AMJ13 vs. Ref	AMN3 vs. AMJ13	AMN3 vs. Ref	AMJ13 vs. Ref	AMN3 vs. AMJ13
3.125	0.0022	0.0086	NS	NS	NS	NS
6.25	0.0008	NS	0.0033	NS	NS	NS
12.5	0.0340	NS	NS	0.0035	NS	0.0035
25	<0.0001	<0.0001	NS	<0.0001	<0.0001	NS
50	<0.0001	<0.0001	NS	<0.0001	<0.0001	NS
100	<0.0001	<0.0001	NS	<0.0001	<0.0001	NS
NS: non-significant						

The results indicate that significant inhibitory effects appeared in the cancer cell lines (AMN3 and AMJ13) at different concentrations, while no significant inhibitory effect appear in normal cell line (Ref) at 48 and 72 hrs.

CONCLUSION

New derivatives of sulfonamide-amino acids ester conjugate using succinamide spacer were synthesized and evaluated for their anticancer activities. The synthesized compounds were characterized and identified by I.R spectra, elemental microanalysis, and 1HNMR study, and it was found that all the results shown good agreements with the proposed chemical structures of the synthesized compounds. A preliminary cytotoxicity assay that evaluated by crystal violet assay indicate that the tested compounds have considerable cytotoxic activity against two cancer cell lines at different concentrations with no significant effect on normal cell line, so the synthesized compounds have selectivity in their action toward cancer cells.

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