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Synthesis and Preliminary Anticancer Evaluation of 6-Mercaptopurine – Methotrexate Conjugate as Possible Mutual Prodrug

Asmaa S. Al-Darraji*,1 and Mohamed H.Mohamed**

*Department of Pharmaceutical Chemistry, College of Pharmacy, University of Baghdad, Baghdad, Iraq

Abstract

Small molecules drug conjugate mutual prodrug design (SMDC) composed of folate and lethal agent conjugate, rigidly bonded via hydrophilic bridge and self immolative disulfide bond ; represent new interesting approaches for cancer treatment , the component of SMDC intended for targeting folate receptor , along with greater conservation of component until reaching the target tumor tissue . The designing and synthesis of compound VI and VIII derived from 6-Mercaptopurine (6-MP) and Methotrexate (MTX) conjugate altogether as mutual prodrugs were processed forward successfully by multistep reaction procedures , and by Thin Layer Chromatography (TLC) for preliminary detection of products and their intermediates, along with their purity. The structures of two final compounds and their intermediates were proclaimed by melting point measurement, infrared spectrometry and ^{1}HNMR analysis given results greatly correspond with theoretical proposed chemical structure of synthesized compounds. Furthermore, cytotoxic activity evaluation on cell line level had been done for two final compounds against human breast tumor cell (MCF-7) and human ovarian tumor cell (SKO-3) types of cancer cell line and the results were confirmed which show greater cytotoxic tumor activity of two final compounds, while compound VI possess optimal activity proportional with increased number of 6-MP molecules.

Keywords: SMDC, 6MP, Methotrexate, Cytotoxic activity.

تصنيع وتقييم مضاد سرطاني اولي لمدمج دوائي من ٦- ميركابتوبيورين والميثوتركسسيت كمقدمات دوائيه محتمله

اسماء صفوان الدراجي*،١ و محمد حسن محمد **

*فرع الكيمياء الصيدلانية، كلية الصيدلة، جامعة بغداد، بغداد، العراق .

الخلاصه

ان تصميم المقترن الدوائي ذو الجزيئات الصغيره كمقدم دوائي ذو فعالية ثنائية يتكون من اقتران الفوليت والماده القاتله ويرتبطان بقوه عن طريق جسر محب للماء ،وثنائي السلفايد الذاتيه التحلل ؛ تمثل مناهج جديدة ومثيرة للاهتمام لعلاج السرطان. مكونات (SMDC) مهياة لاستهداف مستقبلات الفوليت مع الحفاظ وبقوه لمكوناتها الى ان تصل الى الانسجه السرطانيه المقصوده .

تصميم وتصنيع المركبينVI و VIII المشتقين من اقتران ٦- ميركابتوبيورين والميثوتركسيت معا كمقدمات دوائيه ذات فعاليه ثنائيه قد تم بنجاح باتباع طرق التفاعل متعدد الخطوات , وتم مراقبة جميع التفاعلات والتاكد من نقاوة المركبات بواسطة كروماتوغرافيا الطبقة الرقيقة , تركيب المركبين النهائيين مع مركباتهم الوسطية تم اثباته بقياس درجة الانصهار , التحليل الطيفي للاشعة تحت الحمراء , وتحليل الرنين النووي المغناطيسي للبروتون

واعطت نتائج تنطبق بصوره كبيره مع التركيب الكيميائي المفترض نظريا للمركبات المصنعه ، تم تقييم التاثير السمي الخلوي للمركبين النهائيين ضد خلايا سرطان الثدي وخلايا المبيض السرطانيه وتم تثبيت النتائج التي اظهرت درجه عاليه من السميه ضد الخلايا السرطانيه للمركبين النهائيين ،بينما يمتلك المركب VI الفعاليه المثاليه التي تتناسب مع زياده عدد جزيئات ٦- ميركابتوبيورين .

الكلمات المفتاحيه : (SMDC) ، ٦ -ميركابتوبيورين ،الميثوتركسييت ، الفعاليه القاتله للخلايا السرطانيه .

Introduction

6-mercaptopurine, is thiopurine related structurally and belong to antimetabolite group of medication[1] ,it has been discovered in 1948 , published and approved as suitable for human medical use in 1953[2], followed by announcement that it is the safest and relevant effective medication for public health[3]. 6-MP possess limited spectrum against tumor cell, for instance , the use of this medication limited for the treatment of lymphocytic leukemia during acute phase , chronic myeloid leukemia[4], other use apart from cancer treatment when use concomitantly with other medication for treatment auto-immune disease such as crohn's disease [5]and other inflammatory complex disease such as ulcerative colitis [6],specifically 6-mercaptopurine and methotrexate use together during maintains phase of child non-Hodgkin leukemia as the most successful combination for treatment with high curative rates[7] .

[1]Corresponding author E-mail: asmaasafwan2016@gmail.com
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Probably 6-MP possesses many disadvantages that make its use highly confused; first pharmacokinetic consideration , include weak water solubility , poor oral absorption ,also subsequent lower bioavailability , short- half -life less than 1 hour , perhaps slower onset of action ; effect not seen furthest than 3-6 months after initiation of therapy , also in liver convert to inactive metabolite 6- thiouric acid which may revised the balance between the active and inactive metabolite[(^)] , 6-MP associated with serious life-threatening myelosuppression, if untreated, may lead to chronic bone marrow failure exacerbate to secondary cancer[(9)] , also cancer cell shows characteristic rapid resistance shortly after initiation of therapy.[(1٠)]

Prodrug design is chemical modification of parent drug, designed specifically to enhance pharmacokinetic profile and/or to achieve site-specific drug delivery[(11)], the pharmacological activities of parent drug residue are sufficiently retained and recovered only either by enzymatic/non-enzymatic biotransformation.[(12,13)]6-MP find to have greater application in practice, once for improving its water solubility combined with characteristic stability and rapid releasing capability by combining this medication with hyaluronic acid via carbonylvinyl sulfide bridges [(14)] and other achieving targeting action by concentrate drug in one body compartment much greater than the other such as cis-3-(9H-Purin-6-ylthio)acrylic acid (PTA) prodrug targeting tumors with up-regulated GSH levels, and B, *S*-(9H-purin-6-yl)-*l*-cysteine are (prodrug selectively kidney delivered , concentrate 6-MP probably approximate 2.3 to 90 fold higher than plasma and liver) [(15)] prodrugs of the 6-mercaptopurine (6-MP) antitumor agent both need glutathione mediated metabolic activation [(16)] , also another two 6MP derivative 6-[5-pyridine -4-yl- 1, 2, 3, 4-oxadiazole -2-(yl) dithiol]-9H-purine and 9H-purine -6-yl-benzyl dithio carbamate, both possess cytotoxicity level much higher than 6MP, potentiates high inhibitory cytotoxic capability against renal cell and ovarian cell cancer[(17)]. Folate receptor (cancer cell biomarker) [(18)] , FRα and FRβ antagonist show invaluable mean in cancer treatment since these receptor show efficient up regulation in tumor tissues compared with normal tissues ,thus potentiate tumor metastasis , also play a role in cancer development ,and progression[(19)]

Methotrexate, belong to the antimetabolite class of medication of antifolate type and effective chemotherapeutic agent for diverse cancer treatment, closely resemble folic acid structurally property rendering MTX as effective competitive inhibitor for folate as enzymatic co-factor for cellular vitality [(20, 21)].

The work focused on the synthesis of two mutual prodrugs as (SMDC) with molecular weight not exceed 1000dalton, both designed by conjugation of 6MP and MTX through viable enzymatically hydrolyzed amide and significant disulfide bonds.

Materials and Methods

Most of chemicals for research need were supplied by (Hyperchem company/ China); these chemicals include 6-MPand MTX, dicyclo hexyl carbodiinmide (DCC), and 1-hydroxy benzotriazole (HOBT),while cysteine amino acid was supplied by (sinopharm/China), and 4-nitrophenylchloroformate from (Tci /china), purity determination and reaction progress following up realized by thin-layer chromatography (TLC), it was prepared by running diverse mobile phase on stationary phase prepared from coating silica gel F-254(type60) on thin film of aluminum(CHMLAB group/ Spain).The final products and their intermediacy were revealed by attendant iodine vapor or simple irradiation with UV lamp(254nm), featuring melting points of final products and their intermediates were measured by capillary tube method, results accession on electric melting point instrument (Stuart value / England) , and they are uncorrected. Infrared spectra were accomplished by (Biotech / England) utilize KBr disc. ^{1}HNMR analysis performed on (Nanalysis Company / Canada).

Synthesis of methyl cysteinate hydrochloride (compound I) [(22)]

A solution of L-cysteine (41.26 mmol ,5g) in absolute methanol(50 ml), was prepared and cooled to -15 °C, then thionyl chloride (49.2 mmol,3.567 ml) was added drop wise over 10 minute with persistent stirring (the temperature was kept at -15°C) , this mixture was allowed to cool at room temperature before being transferred and refluxed for 3 hrs. Excess solvent was removed under vacuum, then 60ml of diethyl ether was added, and allowed to stand in refrigerator overnight, the bulky product filtered to produce compound I,% yield= 98%, M.P(142°C), **IR** (KBr disc),(υ cm^{-1}): 3398-2511 ($NH3^+$ str),2954(C-H) str. of CH3,2555(S-H) Stretching,1743(C=O) carbonyl ester stretching ,1577 and 1516-1496($NH3^+$) salt a sym. and sym. Bending, 1441 and1404a sym. And sym. (C -H)of CH3 bending, 1246and1205 (C=O-O)a sym. and sym. Stretching , 1068 (C-N) stretching of aliphatic ester.^{1}HNMR (60MHz, DMSOd6 ,δ=ppm): 1.4(1H,s,SH), 3.02(3H,s,CH3) ,2.83,2.67 (2H,q,CH2), 4.13,4.28(1H,d,CH), 8.75(2H,d,NH2).

Synthesis of 7H-purine-6-thiolate potassium salt (compound II) [(23)]

Solution of 6 –mercaptopurine (32.5 mmol ,5g) in 300ml ethanol , and ethanolic solution of KOH (32.5 mmol , 1.82g) was added and stirred for half of an hour with heating followed by further half of an hour at room temperature , the result precipitate was filtered to give compound II,

%yield=92%,M.P=340°C, IR (KBr disc),(υ cm^{-1}):3431cm^{-1}(N-H) stretching of purine ,2931and 2900 cm^{-1}(C-H) a sym.and sym.stretching of imidazole, 1587-1331 cm^{-1}(C=C,C=N) ring stretching ,1331-1254 cm^{-1}(C-N) stretching of imidazole ,904-806 cm^{-1}(C-H) out of plane bending ,706-658 cm^{-1}(C=C) out of plane bending.

Synthesis of1-((7H-purin-6-yl)thio)pyrrolidine-2,5-dione (compound III)

To cold solution of compound II (10.51mmol ,2g) in 100 ml anhydrous DMF , a cold solution of N-bromo succinimide NBS (10.51mmol, 1.87 g) in 20 ml acetone was added with continuous stirring and cooling, the color change from green to orange with complete conversion into clear solution had observed , solution left in ice - bath for 3 hours , followed by overnight stirring at room temperature ,KBr filtered out , cold water was added to filtrate ,the solution kept in refrigerator for 2 days ,precipitate filtered to give compound III, % yield =65%, M.P=195°C, **IR** (KBr disc),(υ cm^{-1}):3464 cm^{-1}(N-H) stretching of purine, 3089and3053 cm^{-1}(C-H) a sym.andsym. Stretching of imidazole ,2976 and 2821 cm^{-1} (C-H) stretching of methylene of succinimide ,1649 cm^{-1}(C=O) stretching of imide, 1236 cm^{-1}(S-N) stretching ,924 cm^{-1}succinimde ring vibration .^{1}HNMR (60MHz, DMSOd6 ,δ=ppm): 2.68,2.83(4H,d,CH2), 8.54 (1H,s,CH) pyrimidine, 8.61 (1H,s,CH) imidazole ,13.75(1H,s,NH).

Synthesis of methyl S-((7H-purin-6-yl) thio) cysteinate hydrochloride salt (compound IV)[24]

Mixture of compound I (4.04 mmol,0.68 g) and compound III (4.04mmol,1.007gm) in 20 ml dimethylformamide (DMF) was refluxed for 3 hours , the mixture then was stirred overnight at room temperature ,then 20ml cold water was added and the resulted precipitate was filtered to give compound IV, %yield=50%,M.P =270°C, **IR** (KBr disc),(υ cm^{-1}):3431 cm^{-1}(N-H) stretching of purine ,3093and3024 cm^{-1} a sym. and sym. (C-H) stretching of imidazole ,2779 cm^{-1} sym. Stretching of cysteine methylene group ,1739 cm^{-1}(C=O) stretching of ester, 1664-1466 cm^{-1}(C=C,C=N) stretching pyrimidine ,1568 cm^{-1}(N-H) bending of cysteine methyl ester ,1377 cm^{-1}(C-H) bending of methyl of cysteine methyl ester, 1221 cm^{-1}(C=O-O)stretching ,509 cm^{-1}(S-S) stretching .^{1}HNMR (60MHz, DMSOd6 ,δ=ppm): 2.67(2H,CH2) of ester ,2.83(1H,CH) of ester,3.62(3H,CH3) of cysteine methyl ester ,8.14(1H,CH) pyrimidine ,8.31(1H,CH) ,13.69(1H,NH) imidazole .

Synthesis of 2-((4-(((4-(l2-azaneyl)-2-(((4-nitrophenoxy) carbonyl) amino) pteridin-6-yl) methyl) (methyl) amino) phenyl) carbamoyl)pentanedioic acid (compound V) [25]

A solution of Para –nitrophenylchloroformate (2.2mmol,0.4434g) was added to cooled solution of Methotrexate (1.1 mmol, 0.5g) and triethylamine (TEA) (2.2 mmol,0.306m) 10ml anhydrous DMF, the mixture was stirred at room temperature for1 hour, then 2equimolar 0.02M HCl added ,and iced-water added andthe resulted precipitate filtered to give compound V .% yield =73%, M.p=109°C, **IR** (KBr disc),(υ cm^{-1}): 3618-2400cm^{-1}(O-H) stretching of carboxyl , ,3116 and 3030 cm^{-1}(C-H)stretching of phenyl rings of 4-NPC and methotrexate respectively , 2966and2941 cm^{-1}(C-H) a sym. Stretching of methyl andmethylene respectively ,1784 and 1662 cm^{-1}(C=O) overlapping of carbamate stretching and carboxylic acid ,1641,1601,1446 cm^{-1}(amide IandII,C=C,C=N)stretching,1520and1350(NO2) asym. and sym. Stretching respectively,1306(O-H) out of plane also(C-O) stretching appear at same region ,1227and1190 cm^{-1}(C=O-OandO-C-C)stretching .^{1}HNMR (60MHz, DMSOd6 ,δ=ppm):1.13(2H,CH2) methylene β to amide, 2.68(2H,CH2)methylene δ to amide ,2.84 (1H,CH) methine α to amide ,3.17(3H,CH3)N-CH3 ,4.77(2H,CH2)methylene next to N-CH3,7.34,7.75,7.9,8.15 (8H,CH) methine of phenyl of 4-NPC ,7.08 ,6.7 ,6.97 ,6.84(4H,CH) methine of benzene MTX, 8.27 (1H,NH) of amide ,8.58(1H,NH) of carbamate ,8.0(1H,CH)of petridine.

Synthesis of 5-methyl 1-(triethyl-l5-azaneyl) (4-(((2 - (3-(3-((7H-purin-6-yl)disulfaneyl)-1-methoxy - 1-oxopropan-2-yl)ureido)-4-(3-(3-((9H-purin-6-y l)disulfaneyl)-1-methoxy-1-oxopropan-2-yl) ureido) pteridin – 6 - yl) methyl) (methyl) amino) benzoyl)glutamate (compound VI)[26]

A solution of compound IV(1.25mmol,0.3847g) in 5ml anhydrous DMF was added to solution of Compound V (0.625mmol,0.5g) and TEA (1.875mmol.,0.2609ml) in 5ml DMF at room temperature, mixture was stirred at room temperature overnight, then 10 ml of distilled water was added, the resulted precipitate was filtered and dried to give compound VI ,%yield =50%, M.P=not measured compound as gel , **IR** (KBr disc),(υ cm^{-1}): 3400 cm^{-1}(N-H) stretching of purine and 2°-amide ,3677-2400 cm^{-1}(N-H)of triethylamine salt,2976and2939 cm^{-1}a sym. stretching of methyland methylene respectively ,1724 cm^{-1} (C=O)stretching of ester,1641cm^{-1} (C=O)stretching of amide and urea,1604 ,1543,1439 cm^{-1}(C=C)also (N-H)bending appear in same region , (C=N) appear in same region ,1336 cm^{-1}(CH2)twisting of cysteine ,1290 cm^{-1}(C-NH) stretching of purine

,1205,1173,1022 cm^{-1}(C=O-O,O-C-C) stretching of ester ,945-700 cm^{-1}(C-H)out of plane bending ,700-509 cm^{-1}(N-H) out of plane . ^{1}HNMR (60MHz, DMSOd6 ,δ=ppm): 1.14(9H,CH3)of triethylaminesalt,1.26 (6H,CH2),1.79 (2H,CH2) methylene βto MTX amide ,2.66 (2H, CH2)δ to MTX amide , 2.77 1H,CH)αto MTX amide, 3.0 (3H,CH3)of N-CH3 ,3.58(CH3,3H),3.82(CH3,3H) of O-CH3 ,4.47 (2H,CH2) methylene next to N-CH3,6.87(1H,CH)of benzene ring ,7.02 (1H,CH) of benzene ring ,7.97,7.91(1H,CH) of benzene ring ,8.12(1H,CH)of pteridine ,8.70 (2H,CH) of pyrimidine, 9.03(2H,CH) of imidazole,9.3 (1H,NH) of amide of MTX ,10.66 (1H,NH) of urea .

Synthesis of (S)-5-((1H-benzo[d][1,2,3]triazol-1-yl)oxy)-2-(4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzamido)-5-oxopentanoic acid(compound VII) [27]

Solution of MTX (1.1mmole ,0.5g) was prepared by dissolving of the defined weight of former in 10ml DMF ,solution then reserved at 0°Cwith constant stirring ,TEA (1.1mmole ,0.15ml) was dropped wise on previous mixture ,followed by DCC(1.1mmol,0.226g), 1-HOBt(1.1mmol,0.148g) addition respectively .Solution then allowed to stand overnight at0°C, followed by addition equimolar of 0.02M HCl then mixture of 30% acetone / diethyl ether was then added to precipitate DCU(dicyclo urea) , filtrate volume diminished under vacuum to give compound VII. % yield =63%,M.P=78-82°C, **IR** (KBr disc),(υ cm^{-1}):3406-3207 cm^{-1} (N-H) stretching and(C-H)stretching of phenyl ring ,3000-2500 cm^{-1}(O-H) free hydroxyl stretching,2929 cm^{-1}(C-H) a sym. stretching of methylene ,1720 cm^{-1},(C=O) stretching of ester ,1657 cm^{-1}(C=O) of carboxylic acid , 1633 cm^{-1} (C=O) of amide stretching ,1603,1556,1508 cm^{-1} (C=N) overlap with (C=C)stretching , 1385 cm^{-1}(C-H)(O-H) bending ,1290-1252 cm^{-1}(C-N) stretching and(C-H) in plane bending .

Synthesis of triethyl-l4-azaneyl N2-(4-(((6,8-diaminonaphthalen-2-yl)methyl)(methyl)amino)benzoyl)-N5-(1-methoxy-1-oxo-3-((4,5,6,7-tetrahydro-9H-purin-6-yl)disulfaneyl)propan-2-yl)glutaminate (compoundVIII) [28]

Compound VII (0.87mmol ,0.5g) was dissolved in 10ml anhydrous DMF , then TEA (2.61mmol ,0.36ml) was added carefully drop by drop to mixture ,then compound IV(0.87mmol,0.273g) was added to previous mixture , the latter had then transferred and reserved at 0°C for 3 days ,followed 2 days at 15°C,(*reaction followed up by TLC),initial precipitation of byproduct did by diethyl ether, then product precipitation by adding 120ml acetone and the resulted precipitate was filtered to give compoundVIII,% yield =65%,M.P=104-110°C, **IR** (KBr disc),(υ cm^{-1}):3600-2150 cm^{-1}(N-H)stretching of ammonium salt ,3357and 3180 cm^{-1}(N-H) a sym.andsym. of amine ,3030 cm^{-1}(C-H)sym**.** stretching of benzene , 1664(C=O) stretching of secondary amide ,1549 cm^{-1}(C=O)stretching of carboxylate anion ,1643and1603(amide IandII)also (NH2)bending appear in same region ,1549 cm^{-1} ,1508,1475,1446 cm^{-1}(C=C)stretching of benzene ,1398 cm^{-1}(C-H) methylene scissoring overlap with methyl scissoring also,(C=N) appear in same region ,1334 cm^{-1}(C-H) methylene twisting ,1254,1201(C=O-O)and(C-O-C)of ester stretching ,1078 cm^{-1}(C-N)stretching of aromatic amine ,1034and764 cm^{-1}(C-H) in plane and out of plane bending ,852and833 cm^{-1}(N-H) wagging ,704-606 cm^{-1}(N-H)amide out of plane bending .^{1}HNMR (60MHz, DMSOd6 ,δ=ppm):1.14 (9H,CH3)methyl of triethylamine MTX, 1.26 (6H,CH2) of TEA ,2.45 (2H,CH2), 2.68(2H,CH2)methylene σ to amide , 2.8 (1H,CH) α to amide, 3.05 and 3.17(6H,CH3) N-CH3 and O-CH3,3.39 (2H,CH2)methylene next to N-CH3 ,6.83(1H,NH) of amide of MTX,7.63(1H,CH)of benzene ,7.77(1H,CH) of benzene ,8.12(1H,NH) of pyrimdine ,8.31(1H,CH)of imidazole ,8.54(1H,CH)of pteridine.

Cytotoxicity assay of compound VI, VIII [29]

The cytotoxicity were determined for both targeting mutual prodrug (VI,VIII) by utilization MTT assay method on SKO-3andMCF-7malignant cell ,and this study was performed in Iraq Biotech company .While the results was compared with 6MP as standard for our work ,the antitumor potency was dedicated on measurement of cell viability (MTT)assay, cell line were labeled on $1x10^4$cells/well and by using 96 wells plates andafter 24hrs , compounds with their consecutive standard tested at different conc. , 72hrs time need for close contact between cell and samples before viability measurement ,then their medium replace by 28µl of 2 mg/ml solution of MTT and incubation for furthest 2.5hrs at 37°C, then MTT solution removed ,while the rest crystals collected and incubated after solubilization in DMSO for quarter of an hr at 37°C with stirring. Absorbency was determined on micro-plate's reader (492nm), and percent of cytotoxicity calculated as in following equation

Cytotoxicity% = (A-B/A)*100

Where **A, B** are the optical density of control and the optical density of test respectively. ([30])

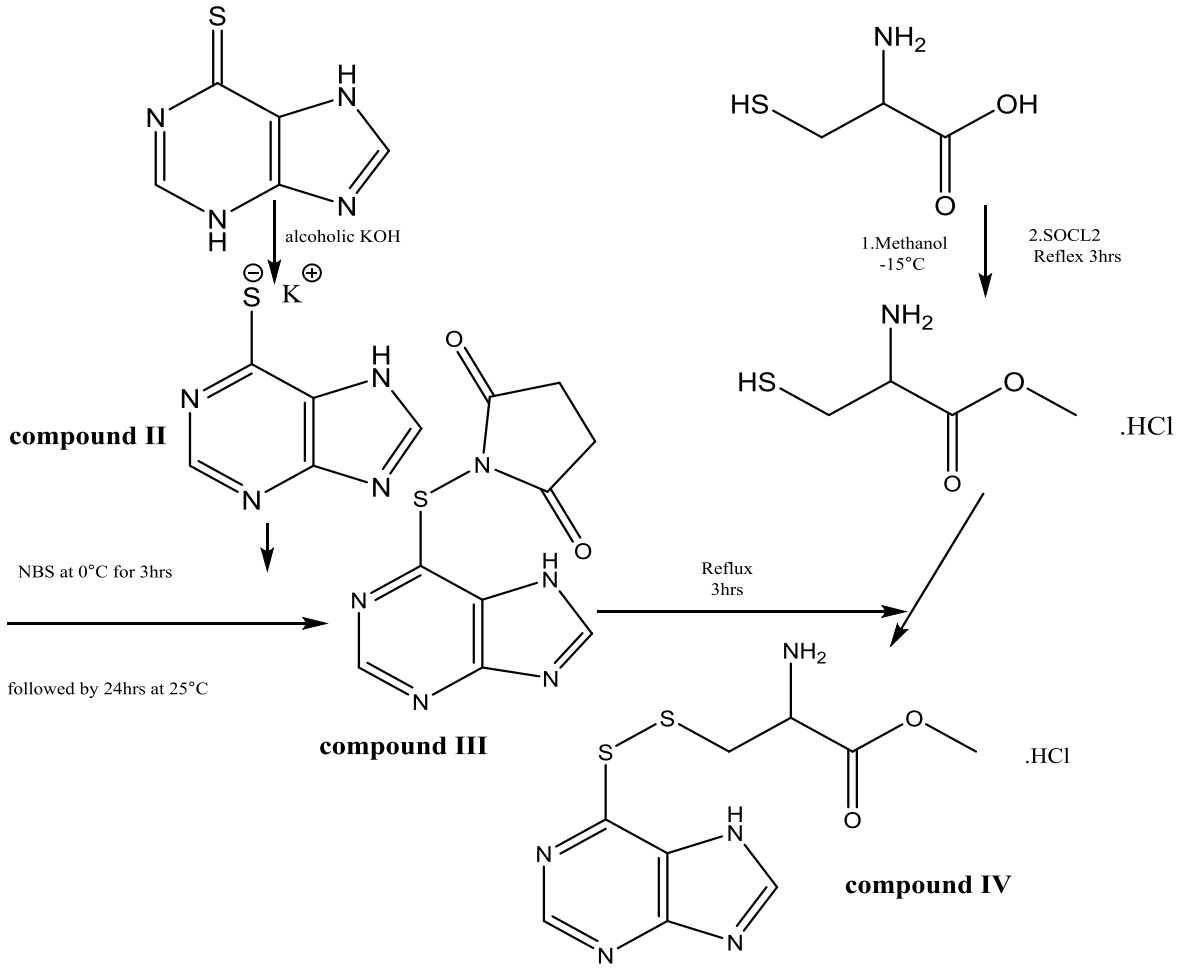
Half maximal inhibitory concentration (IC50)

The quantitive values represent in molar concentration of drug need to inhibit intended biological process by half.[31] The IC50 of standard and compound VI and VIII against MCF-7 and SKO-3 all mentioned in tables (1) and (2).

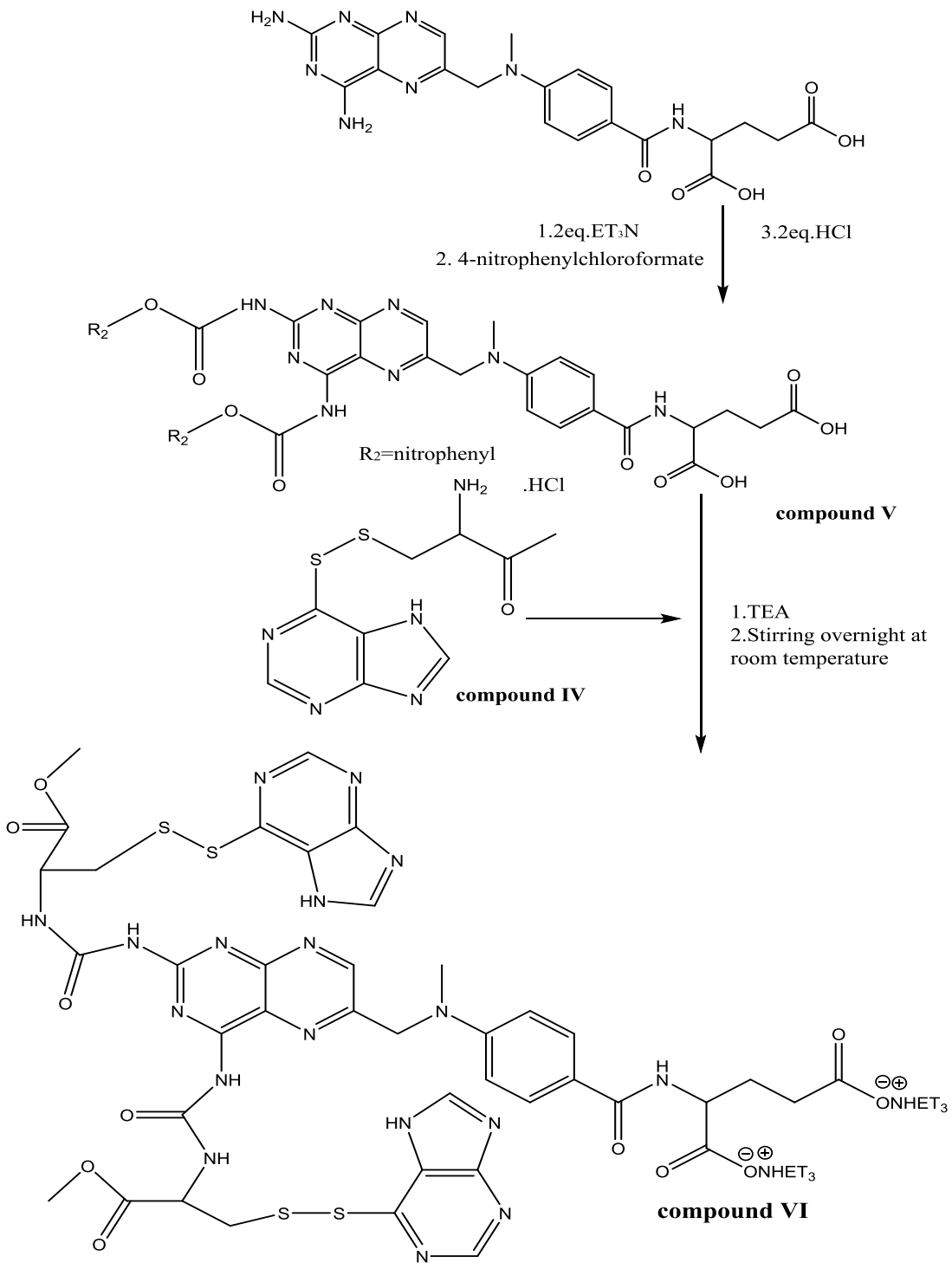
Results and Discussion

Chemistry

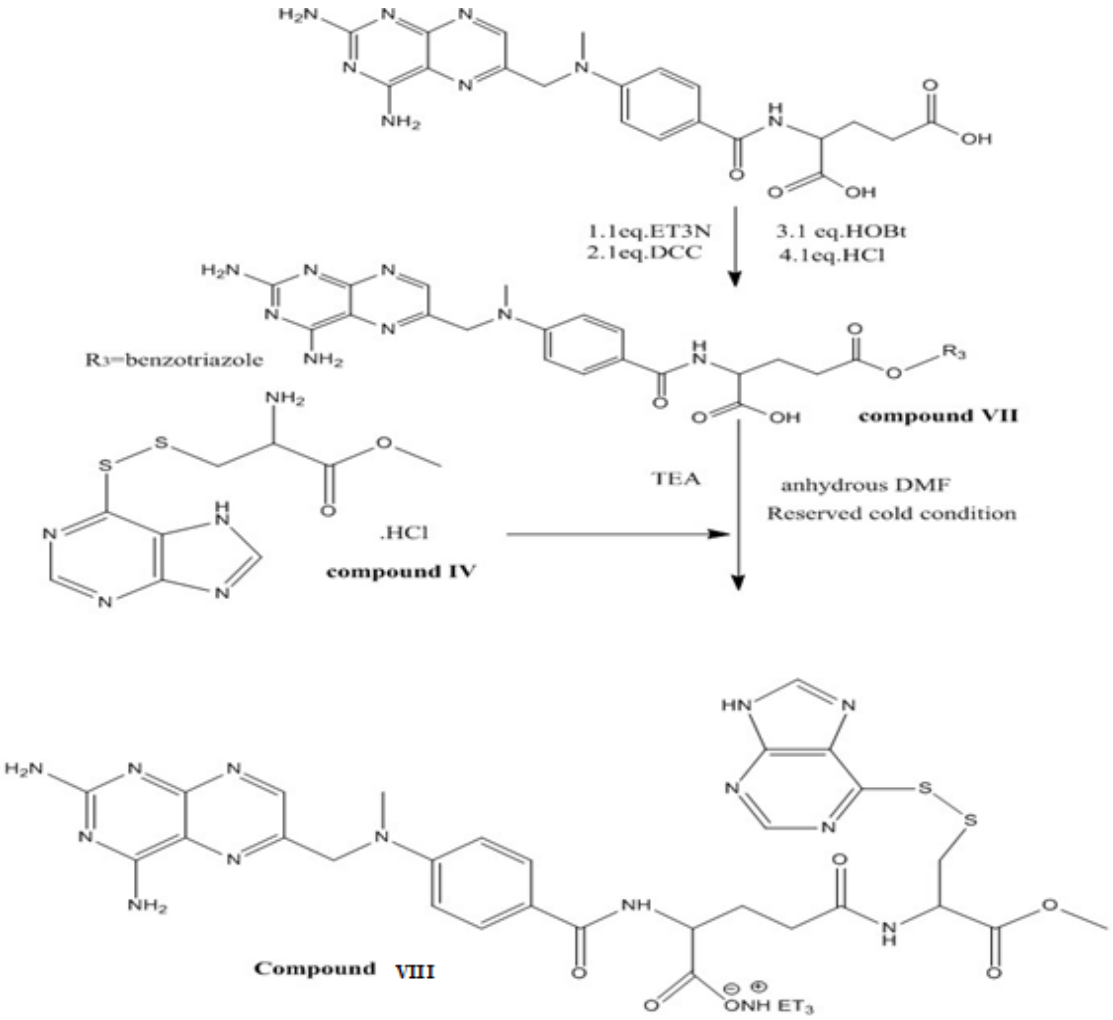
The synthesis of compound I is a process of protection of cysteine amino acid, and this is done by esterification on carboxyl group ,activation initially by thionyl chloride (SOCl2) , these in the presence of anhydrous methanol lead to the substitution of methyl moiety on carboxyl and (compound I) produce. Next step is to enhance of nucleophilicity of thiol group of 6-MP and these criteria had been achieved by converting of thiol group primarily into its corresponding potassium salt ,followed by thiolate substitution on electrophile N-bromosuccinimde ,and (compound III) liberate .Then compound I react with compound III at neutral PH and in slow rate reaction process to produce compound IV. In other hand, MTX is protected on its two 1°amine group by carbamate formation by reaction of MTX in anhydrous DMF with 2equimolar of 4-nitrophenylchloroformate to produce compound V, same dry condition in same solvent required for compound VI synthesis by reaction of compounds V and IV with stirring at 25°C for 24 hrs. On other hand, MTX protected on its γ carboxyl and again in anhydrous DMF by reacting it with 1-hydroxbenzotriazole, process by carbodiimide coupling and compound VII produce. Finally, to produce compound VIII reaction of compounds IVand VII take place in condition in which reaction retained at lower temperature and in dry medium.



Scheme (1) Synthesis of compounds (I-IV)



Scheme (2) Synthesis of mutual prodrug VI.



Scheme (3) Synthesis of mutual prodrug VIII

Cytotoxic activity

Cytotoxic assay of the tested compound against MCF-7 type of tumor cell

Herein , % of cytotoxicity,IC50% results of standard and the two synthesized VI,VIII were shown in figure 1, while statistical data of them shown in table1.

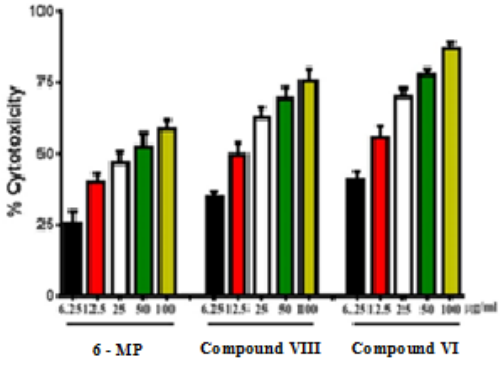


Figure (1) Cytotoxic activity of 6MP, compound VI, VIII against MCF-7 cell line.

Table (1) Cytotoxic activity of 6-MP, compound VIII and compound VI against MCF-7 cancer cell (*Mean ±SEM is mean and standard error of mean, P value is probability value, NS: non-significant value, ** significant value)**

compound number	IC50%	Inhibitory Concentration µg/ml	Cytotoxicity Mean ± SEM	*P* value
6-MP	29.72	6.25 (µg/ml)	25.67 ±2.404	NS
		12.5(µg/ml)	40.00±1.732	<0.0001(****)
		25(µg/ml)	46.67±2.333	<0.0001(****)
		50(µg/ml)	52.33±2.848	<0.0001(****)
		100(µg/ml)	58.67±1.85	<0.0001(****)
Compound VIII	13.51	6.25 (µg/ml)	34.67±1.202	<0.0001(****)
		12.5(µg/ml)	49.67±2.404	<0.0001(****)
		25(µg/ml)	62.67±2.028	<0.0001(****)
		50(µg/ml)	69.33±2.333	<0.0001(****)
		100(µg/ml)	75.67±2.333	<0.0001(****)
Compound VI	10.44	6.25 (µg/ml)	41.00±1.528	<0.0001(****)
		12.5(µg/ml)	55.67±2.333	<0.0001(****)
		25(µg/ml)	70.00±1.732	<0.0001(****)
		50(µg/ml)	77.67±1.202	<0.0001(****)
		100(µg/ml)	87.00±1.155	<0.0001(****)

Cytotoxic assay of the tested compounds against SKO-3 type of tumor cell

% of cytotoxicity, IC50% results of standard and the two synthesized VI, VIII were shown in figure 2, while statistical data of them shown in table 2.

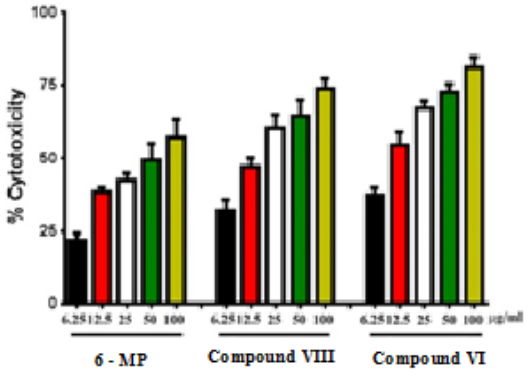


Figure (2) Anti-proliferative activity of 6MP, compound VI, VIII against SKO-3 cell line

Table (2) Statistical results of cytotoxic activity of 6-MP, compound VIII and compound VI against SKO-3 cancer cell (Mean ±SEM is mean and standard error of mean, P value is probability value, NS: non-significant value, ** significant value)**

compound number	IC50%	Inhibitory Concentration µg/ml	Cytotoxicity Mean ± SEM	*P* value
6-MP	41.14	6.25 (µg/ml)	21.07 ±1.453	NS
		12.5(µg/ml)	38.33± 0.8819	<0.0001(****)
		25(µg/ml)	42.33 ±1.453	<0.0001(****)
		50(µg/ml)	49.33± 3.180	<0.0001(****)
		100(µg/ml)	57.00± 3.51	<0.0001(****)
Compound VIII	16.23	6.25 (µg/ml)	32.00± 2.082	<0.0001(****)
		12.5(µg/ml)	47.00± 1.732	<0.0001(****)
		25(µg/ml)	60.33 ±2.603	<0.0001(****)
		50(µg/ml)	64.33± 3.283	<0.0001(****)
		100(µg/ml)	73.67 ±2.028	<0.0001(****)
Compound VI	11.17	6.25 (µg/ml)	37.33± 1.453	<0.0001(****)
		12.5(µg/ml)	54.33 ±2.603	<0.0001(****)
		25(µg/ml)	67.00± 1.528	<0.0001(****)
		50(µg/ml)	72.33 ±1.764	<0.0001(****)
		100(µg/ml)	81.00 ±2.082	<0.0001(****)

The results of cell line study against MCF-7andSKO-3 malignant cell show that compound VI possess the optimal inhibitory activity compared to compound VIII and this activity is at optimal value versus MCF-7 compared to SKO-3types of cancer cell, while compound VIII have moderate potency against same cancer cell mentioned earlier, and both synthesized compounds have anticancer potency much higher than standard 6-MP.

Conclusion

Two new mutual prodrugs were synthesized, both by conjugation of 6-MP and MTX through viable amide bond and reversible disulfide bond on relatively different positions relative to MTX molecules. These compounds were also evaluated on cell line level and results compared with standard and revealed that these prodrugs possess cytotoxic concentration against selected cancer cell much higher compared to standard.

IR, ^{1}HNMR analysis results gave certainty that required compounds were obtained, while in vitro preliminary cytotoxic activity evaluation by MTT assay performance leads to conclusion that synthesized compounds have maximal potency on cancer cell compared to standard at much lower dose. Lead in conclusion that lower doses of two prodrugs may give same or evenly higher response compared to standard drug.

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References

1. Wyman KW, Puzanov I, Hande KR. Purine Antimetabolites. Encyclopedia of Cancer (2nd ed.). 2002 : 515-525.
2. Sternbach L. 6-Mercaptopurine, Librium's discoverer. Chemical and Engineering News . 2005; 83(25): 1-6.
3. Sahasranaman S, Howard D, Roy S. Clinical pharmacology and pharmacogenetics of thiopurines. Eur J Clin Pharmacol. 2008 ;64(8):753-767.
4. Bosch V, Suciu S, Thyss A, Bertrand Y , Norton L, Mazingue F. Value of intravenous 6-mercaptopurine during continuation treatment in childhood acute lymphoblastic leukemia and non-Hodgkin's lymphoma: final results of a randomized phase III trial (58881) of the eortc. Leukemia. 2005 ;19(5): 721–726.
5. Seidman EG. 6-Mercaptopurine in maintaining remission in Crohn's disease: An old friend becomes a new hero. AGAJournals. 2000 ;119(4): 1158–1160
6. 6.Goldstein ES, Marion JF, Present DH.6-Mercaptopurine is effective in Crohn's disease without concomitant steroids. Inflamm Bowel Dis . 2004;10(2):79-84.
7. Nielsen SK, Frandsen SN, Nersting TL, Jacob . Mercaptopurine/Methotrexate Maintenance Therapy of Childhood Acute Lymphoblastic Leukemia: Clinical Facts and Fiction. J Pediatr Hematol Oncol. 2014;(36): 03–517.
8. Nilsen H, vainer Band Rask-Madesen J. Review article: the treatment of in inflammatory bowel disease with 6-mercaptopurine or azathioprine. Aliment Pharmacol Ther. 2001 ;15(11):1699-1708.
9. Rudin S, Marable M , Huang RS. The Promise of pharmacogenomics in reducing toxicity during acute lymphoblastic leukemia maintance treatment . Genomics Proteomics.2017 ;15(2):82-93.
10. Hagner N, Joerger M. Cancer chemotherapy: targeting folic acid synthesis. Cancer Manag Res. 2010; v(2): 293–301.
11. Mohammed MH, Al-Karagully HJ. Cephalothin as a carrier of 6-mercaptopurine for targeting cancer tissues .Iraqi J Pharm Sci.2008; (17):32-40.
12. Ruzza P, and Calderan A .Glutathione Transferase (GST)-Activated Prodrugs. Pharmaceutics. 2013; 5(2): 220–231.
13. Rautio J, Meanwell NA, Hageman MJ. The expanding role of prodrugs in contemporary drug design and development .Nat Rev Drug Discov. 2018;17 (8):559-587.
14. Qiu J , Cheng R, Zhang J, Sun H, Deng C , Meng F , and Zhong Z. Glutathione-Sensitive Hyaluronic Acid-Mercaptopurine Prodrug Linked via Carbonyl Vinyl Sulfide, A Robust and CD44-Targeted Nanomedicine for Leukemia. Biomacromolecules. 2017; 18 (10): 3207–3214.
15. Gunnarsdottir S and Elfarra AA. Glutathione-Dependent Metabolism ofcis-3-(9H-Purin-6-ylthio) acrylic Acid to Yield the Chemotherapeutic Drug 6-Mercaptopurine: Evidence for Two Distinct Mechanisms in Rats. Journal of Pharmacology and Experimental Therapeutics . 1999; 290 (3): 950-957.
16. Ruzza P and Calderan A. Glutathione Transferase (GST)-Activated Prodrugs. Pharmaceutics. 2013 ; 5(2): 220–231.
17. Mohammed MH and Taher MA. Synthesis of new two derivatives of 6-mercaptopurine [5-pyridine -4-yl- 1, 2, 3, 4-oxadiazole -2-(yl)dithiol]-9H-purine (38)and 9H-purine -6-yl-benzyl dithio carbamate (45) with cytotoxicity results from national cancer institute anticancer drug .International Journal Of Pharmaceutical Science 2012;3(8): 1000-1009.
18. Kurahara H. Clinical significance of folate receptor β-expressing tumor-associated macrophages in pancreatic cancer. Ann Surg Oncol. 2012 ;19(7):2264-2271 .
19. Shen J, Hu Y, Putt KS, Singhal S, Han H , Visscher DW, Murphy LM, and Low PS. Assessment of folate receptor alpha and beta expression in selection of lung and pancreatic cancer patients for receptor targeted therapies. Oncotarget. 2018 ; 9(4): 4485–4495.
20. Hagner N, Joerger M. Cancer chemotherapy: targeting folic acid synthesis. Cancer Manag Res. 2010;v(2): 293–301.
21. Nadhem SA, Mohammed MH. Design ,synthesis ,characterization ,and preliminary anticancer study of Methotrexate Silibinin conjugates . Iraqi J Pharm Sci. 2015;Vol.24(1):74-84.
22. Chalker JM, Gunnoo SB, Boutureira O, Gerstberger SC, Fernández-González M , Gonçalo J , Bernardes L . Methods for converting cysteine to dehydroalanine on peptides and proteins. Chemical Science. 2011;(2): 1666-1676 .

23. Gupta V and Carroll KS. Profiling the reactivity of cyclic C-nucleophiles towards electrophilic sulfur in cysteine sulfenic acid. Chemical Science. 2016; 7(400):1-19.
24. Witt D ,Recent Developments in Disulfide Bond Formation. Synthesis . 2008 :2491-2509.
25. Aziz J and Hamze A, Protecting Groups. Tetrahedron. 1996; 52: 1-46.
26. Chaturvedi D, Recent development on the carbamation of Amines . Current Organic Chemistry.2011;15: 1593-1624.
27. Montalbetti CAGN and Falque V. Amide bond formation and peptide coupling. Tetrahedron 2005 ; v(61) :10827–10852
28. Valeurw E and Bradley M. Amide bond formation: beyond the myth of coupling reagents. Chemical Society Reviews. 2008; 38: 606–631.
29. Sulaiman GM, Jabir MS, Hameed AH. Nanoscale modification of chrysin for improved of therapeutic efficiency and cytotoxicity. Arificial cells, Nanomedicine, and biotechnology 2018; 31: 1-13.
30. Al-Shammari AM, Salman MI, Saihood YD, Yaseen NY, Raed K, Shaker HK, Ahmed A, Khalid A, Duiach A. In vitro synergistic enhancement of newcastle disease virus to 5-fluorouracil cytotoxicity against tumor cells. Biomedicines 2016 Jan 29; 4(1): 3.
31. Aykul S, Martinez-Hackert E. Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis. Anal Biochem. 2016; 508: 97-103.