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

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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-Mercaptopururine (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 ¹HNMR analysis given results greatly correspond with theoretical proposed chemical structures. 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-mercaptopururine (SMDC) molecules.

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

Introduction

6-mercaptopururine, is thiopurine related structurally and belong to antimetabolite group of medication, it has been discovered in 1948, published and approved as suitable for human medical use in 1953, followed by announcement that it is the safest and relevant effective medication for public health. 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, other use apart from cancer treatment when use concomitantly with other medication for treatment auto-immune disease such as crohn’s disease and other inflammatory complex disease such as ulcerative colitis, specifically 6-mercaptopururine and methotrexate use together during maintains phase of child non-Hodgkin leukemia as the most successful combination for treatment with high curative rates.
Probably 6-MP possesses many disadvantages; 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, also cancer cell shows characteristic rapid resistance shortly after initiation of therapy.\(^{(1)}\)

Prodrug design is chemical modification of parent drug, designed specifically to enhance pharmacokinetic profile and/or to achieve site-specific drug delivery\(^{(1)}\), the pharmacological activities of parent drug residue are sufficiently retained and recovered only either by enzymatic/non-enzymatic biotransformation\(^{(11,12)}\); 6-MP find to have greater application in practice, once improving its water solubility combined with characteristic stability and rapid releasing capability by combining this medication with hyaluronic acid via carboxyvinyl 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, 5-(9H-purin-6-yl)-l-cysteine (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-MP and MTX, dicyclo hexyl carbodiimide (DCC), and 1-hydroxy benzo triazole (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 (Biotec / 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 60 ml 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), \(\text{IR (KBr disc),} (\text{v cm}^{-1})\): 3398-2511(\(\text{NH}^+\) str,2954(C=H) str of CH3,2555(S=H) Stretching,1743(C=O) carbonyl ester stretching ,1577 and 1516(NH3+ salt a sym. and sym. Bending, 1441 and1404a sym. And sym. (C=H)of CH3 bending, 1246and1205 (C=O=O=O) a sym. and sym. Stretching , 1068(C-N) stretching of aliphatic ester,\(\text{^1}HNMR\) (60MHz, DMSO) (\(\delta\) 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), 8.75,8.14ppm (2H,q,CH2), 14.3,14.28(1H,d,CH), 8.75,8.14ppm (2H,d,NH2).

### Synthesis of 7H-purine-6-thiolate potassium salt (compound II)\(^{(23)}\)

Solution of 6–mercaptopurine (32.5 mmol ,5g) in 300 ml 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,
The resulted precipitate was filtered to give compound III, % yield =65%, M.P=195°C. IR (KBr disc),(ν cm⁻¹):3464 cm⁻¹(N-H) stretching of purine, 3089 and 3053 cm⁻¹(C-H) stretching of imidazole, 1649 cm⁻¹(C=O) stretching of imidazole, 1236 cm⁻¹(S-N) stretching, 924 cm⁻¹ (C=C, C=N) stretching of imidazole, 825 cm⁻¹ (C=O) stretching of succinimide, 753 cm⁻¹ (C=N) stretching of imidazole, 650 cm⁻¹ (C-N) stretching. 

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

Mixture of compound I (4.04 mmol,0.68 g) and compound III (4.04 mmol,1.007g) 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),(ν cm⁻¹):3431 cm⁻¹(N-H) stretching of purine, 3093 and 3024 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, 1646-1466 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 .¹HNMR (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-(12-azanelyl)-2-(((4-nitrophenoxy) carbonyl amino) pteridin-6-yl) methyl) (methyl) amino) phenyl carbamoyl)pentanedioic acid (compound V)²⁵

A solution of Para – nitrophenylchloroformate (2.2 mmol,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 ice-water added andthe resulted precipitate filtered to give compound V, % yield =73%, M.p=109°C. IR (KBr disc),(ν cm⁻¹): 3618-2400 cm⁻¹(O-H) stretching of carboxyl , 3116 and 3030 cm⁻¹(C-H) stretching of phenyl rings of 4-NPC and methotrexate respectively, 2966 and 2941 cm⁻¹ (C-H) a sym. Stretching of methyl and methylene respectively, 1784 and 1662 cm⁻¹(C=O) overlapping of carbamate stretching and carboxylic acid, 1614,1601,1446 cm⁻¹ (amide Iand II,C=C,C=N) stretching,1520 and 1350(NO2) asym. and sym. Stretching respectively, 1306(O-H) out of plane also(C-O) stretching appear at same region ,1227 and 1190 cm⁻¹( C=O-Oand O-C) stretching. .¹HNMR (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, 7.9,8.15 (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,CH) of carbamate, .8.0(1H,CH) of pteridine.

Synthesis of 5-methyl 1-(triethylaminolyl) 4-( ((2 - (3-(3-((7H-purin-6-yl)disulfanyl)-1-methoxy -1-oxopropan-2-y lureido)-4-(3-(3-(9H-purin-6-yl disulfanyl)-1-methoxy-1-oxopropan-2-y lureido) pteridin – 6 - yl ) methyl ) (methyl ) amino ) benzoyl)glutamite (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),(ν cm⁻¹): 3400 cm⁻¹(N-H) stretching of purine and 2°-amide, .3677-2400 cm⁻¹(N=O) of triethylamine salt,2976 and 2939 cm⁻¹ a sym. stretching of methyl and methylene respectively, .1724 cm⁻¹(C=O) stretching of ester, 1614 cm⁻¹ (C=O) stretching of amide and urea, 1604,1543,1439 cm⁻¹ (C=O)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
1,1205,1173,1022 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. ¹H NMR (60MHz, DMSO-d₆, δppm): 1.14(9H,CH₃) of triethylaminesalt,1.26(6H,CH₂),1.79(2H,CH₂) methylene β to MTX amide, 2.66(2H,CH₂) γ to MTX amide, 2.77(1H,CH) onto 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-benzol[d][1,2,3-triazol-1-yl)oxy]-2-(4-((2,4-diaminopteridin-6-yl) methyl)(methylamino)benzamido)-5-oxopentaonic 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°C with constant stirring. TEA (1.1mmole,0.15ml) was dropped wise on previous mixture, followed by DCC(1.1mmol,0.226g), 1HOBt(1.1mmol,0.148g) addition respectively. Solution then allowed to stand overnight at 0°C, followed by addition equimolar of 0.02M HCl then mixture of 30% acetone/ diethyl ether was then added to precipitate DCC (dicyclohexylurea), filtrate volume diminished under vacuum to give compound VII. % yield = 63%,M.P=78-82°C, IR (KBr disc),(ν cm⁻¹):3406(ν(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-14-azaneyl N2-4-(((6,8-diaminophenalen-2-yl)methyl)(methylamino)benzoyl)-N5-(1-methoxy-1-oxo-3-((4,5,6,7-tetrahydro-9H-purin-6-yl)diazasulanyl)propan-2-yl)glutamate (compound VII) (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),(ν cm⁻¹):3600-2150 cm⁻¹(N-H) stretching of ammonium salt, 3357 and 3180 cm⁻¹(N-H) a sym. and sym. 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, 1643 and 1603 (amide I and II) also (NH) 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, 1034 and 764 cm⁻¹(C-H) in plane and out of plane bending, 852 and 833 cm⁻¹(N-H) wagging, 704-606 cm⁻¹ (N-H) amide out of plane bending. ¹H NMR (60MHz, DMSO-d₆, δppm): 1.14 (9H,CH₃)methyl of triethylamine MTX, 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, 3.92(2H,CH₂)methylene next to N-CH₃, 6.83(1H,NH) of amide of MTX, 7.63(1H,CH) of benzene, 7.77, 7.18(1H,CH) of benzene, 8.12(1H,NH) of pyrimidine, 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-3 and MCF-7 malignant 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 1×10⁶ cells/well and by using 96 wells plates and after 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 microplate'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 quantitative 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-bromosuccinimide, 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 IV and 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 table 1.

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

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-3 types 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, ¹HNMR 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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