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Research Article

Design and development of aliphatic amino acid-cholesterol biomolecular scaffold as anticancer conjugates.

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ABSTRACT

We have developed lipoprotein macromolecular motif to target multiple type of cancerous cells. These scaffold moieties linked with anticancer agents for targeting release at specific site. Biomolecular network increases cellular penetration, specificity and efficacy. Molecular motifs containing these agents are readily degradable by enzymatic cleavage. Structural functionalities of these modified molecules generate response against cancerous cells. Lipids and protein conjugates improve drug delivery towards target tissues. Bioactive lipoprotein exerts inhibitory effect for progressing tumor tissues. Lipid-protein bioconjugates interact with tumor tissue proteins selectively for reducing toxicity of antitumor agents. Complexation of cholesterol with bioactive aliphatic amino acid yields complex scaffold possessing anticancer activity. Reaction was conducted using dicyclohexyl carbodiimide (DCC) and 4-dimethylamino pyridine (DMAP) in pyridine solvent. Developed conjugates were characterized by using TLC, IR, NMR and HRMS studies. Conjugates were screened for anticancer activity by using MTT assay for human lung cancer (A549), liver hepatocellular carcinoma (HepG2), Human colon cancer (HT-29), Breast carcinoma (MCF-7), Glioblastoma cell lines (U87 MG).All molecular motifs exhibited remarkable antitumor activity against specified cell lines. Non-toxicity towards normal mouse fibroblast (L-929) is the promising feature of synthetic biomolecular scaffold which indicates selectivity of molecular complexes on tumor tissues. Current synthetic protocol can be used for development of biochemical molecular motif with high specificity, selectivity and antitumor potential.

KEYWORDS

Cholesterol, aliphatic amino acids, anticancer, bioconjugates.

1. INTRODUCTION

Lipids and amino acids are bioactive macromolecules possessing potential to interact with cellular membrane and receptor surfaces. These biomolecular networks assist cancer treatment by interaction with chemotherapeutic agents. Biomolecular scaffold enhance efficacy of drug along with residence time in tumor tissues. Molecular entities reduce side effects and toxicity of drug. These types of bio complexes modulate resistance shown by cancerous tissue towards drug and maintain effectiveness of therapeutic agents [1,2,3,4].

Biomolecular scaffolds of anticancer agents achieve controlled release and drug targeting. Macromolecular anticancer conjugates found to be more effective as compared to parent drug. Cancerous cell require cholesterol in large extent for growth and progression. Lipoprotein receptor interacts with enzymatic system of cell. Cholesterol based esters found to be suitable target molecules for chemotherapy [5]. Reported research studies exhibited development of amino acid based liposomal system as effective drug delivery tool. Amino acid containing lipid construct shows potential anticancer effect on breast cancer tissues. These bioactive carrier moieties increase permeation of drug through liposomal membrane. These bio entities interact with cellular membrane and exert potential cytotoxic action [6].

Anticancer agents are conjugated with lipoamino acid based derivatives for exerting potent cytotoxic effect. Therapeutic profile and pharmacokinetic properties of drug can be improved using bioactive molecular system based complexation strategies [7]. Linking of lipoamino acid to anticancer scaffold enhances accumulation of drug with tumor tissues. Lipoidal-peptide based system increases cellular uptake of drug. Reduction of doses and frequent tumor protein interaction is possible using biomolecular design. These bio constructed moieties makes drug potent and enhances therapeutic effect. Lipoprotein based molecular entities are effective against sensitive and resistant cell lines [8]. Long alkyl chain containing lipoprotein moieties interact with lipid bilayer membrane for solubility enhancement. Non polar alkyl chain and polar functional entities enhances bioavailability. These biomolecular entities are compatible for targeted delivery. Motifs containing these molecular functionalities interact with serum proteins and lipid bilayer effectively. Lipoprotein coupled complexes increases drug affinity towards bio membrane [9]. Biomacromolecular agents increase tumor tissue permeability and retention of anticancer agents in target tissues. Cross linking of antitumor agents with tumor tissues improves permeability and retention of drug in tumor tissue. Additionally, these bio adduct system restrict growth of tumor tissues and interferes with cellular mechanism. Chemotherapy restricted growth of tumor tissues and toxic effects associated with system. Biological macromolecules effectively penetrate for improving drug localization in tissues [10].

Based on aforementioned discussion chemically modified lipoprotein conjugates were developed by cross linking of cholesterol with amino acids like alanine, cysteine and aspartic acid using DCC and DMAP to form to form corresponding ester conjugates. Conjugates were confirmed by using IR, ^{1}H NMR and HRMS. Developed lipoprotein conjugates show improved activity against specified tumor cell line and no toxicity against normal cancer cell lines in MTT assay.

2. MATERIALS AND METHODS

Fourier transform infrared spectra were recorded on a Bruker FT-IR spectrometer. ^{1}H NMR was recorded on a Bruker AC (300 MHz for ^{1}H NMR) and Bruker AC (400 MHz for ^{1}H NMR) spectrometer using DMSO- d_6 as solvent and tetramethylsilane (TMS) as an internal standard. High Resolution Mass Spectra were recorded on a Agilent HRMS 6200 Q-TOF Spectrophotometer., L-Alanine, L-Cysteine, L-Aspartic acid were purchased from Sigma Aldrich.Cholesterol, N,N-Dicyclohexylcarbodimide (DCC),4-Dimethylamino pyridine(DMAP)), Pyridine was purchased from Sigma Aldrich Reactions were monitored by TLC (precoated silica gel plate F254, Merck) using UV light, iodine vapours. Column chromatography was performed by using dry Merck based Silica gel (230-400 mesh) grade. Solvents were dried over Sodium sulfate as drying agents and freshly distilled prior to use. Ultrapure water (Milli-Q, 18M·Ω) was used at all times in the experiment. In vitro cytotoxicity study was carried out using U-87, MCF-7, Hep-G2, HT-29 and A549 cell lines. Cell lines were procured from NCCS Pune.

2.1. Procedure for synthesis of Cholesterol-Alanine and Cholesterol-Cysteine scaffold [11]

Reaction was carried out with modification in procedure reported by M. Owais et al. Aliphatic amino acids (1.2mmole) were dissolved in 5 ml pyridine. Frequently, 0.54 mmol of activating agents N, N-dicyclohexylcarbodiimide (DCC) was added and the reaction mixture was stirred for 30 min at room temperature. Afterwards, 1.2mmol of cholesterol was dissolved and reaction mixture was processed for esterification in the presence of catalyst, 0.182 mmol 4-dimethylaminopyridine (DMAP).The reaction mixture was stirred for a period of 12 hr at room temperature. Reaction completion was observed by TLC in (7:3 v/v) DCM: MeOH solvent system. Solvents from reaction mixture were removed using rotatory evaporator under vacuum. Dried product was dissolved in DCM and extracted with acetic acid, brine, 5% $NaHCO_3$ solution and finally washed with water. The organic phase was passed over Sodium sulfate was filtered, and evaporated to dryness under vacuum. Column chromatography was performed for purification of conjugate using DCM: MeOH in (7:3 v/v) proportion as a solvent system.

2.2. Procedure for synthesis of Cholesterol-Aspartic acid scaffold [11]

Reaction was carried out with modification in procedure reported by M. Owais et al. Aspartic acid (1.2mmole) was dissolved in 5 ml pyridine. Frequently, 1.08 mmol of activating agents N, N-dicyclohexylcarbodiimide (DCC) was added and the reaction mixture was stirred for 30 min at room temperature. Afterwards, 2.4 mmol of cholesterol was dissolved and reaction mixture was processed for esterification in the presence of catalyst, 0.364 mmol 4-dimethylaminopyridine (DMAP).The reaction mixture was stirred for a period of 12 hr at room temperature. Reaction completion was observed by TLC in (7:3 v/v) DCM: MeOH solvent system. Solvents from reaction mixture were removed using rotatory evaporator under vacuum. Dried product was dissolved in DCM and extracted with acetic acid, brine, 5% $NaHCO_3$ solution and finally washed with water. The organic phase was passed over Sodium sulfate was filtered, and evaporated to dryness under vacuum. Column chromatography was performed for purification of conjugate using DCM: MeOH in (7:3 v/v) proportion as a solvent system.

2.3. Preparation of Cholesterol-Alanine conjugates

Off white crystals, yield 90%; m.p.195-197^{0}C (decomp.); R_f (DCM/MeOH,7:3):0.58; FTIR (ATR,υ cm^{-1}): 699,772, 890 (C-H alkene), 1055, 1080 (C-O), 1490 (C=C),1743 (ester C=O), 2940 (C-H aliphatic), 3260(NH_2).
^{1}H NMR (400MHz, DMSO-d_6, ppm):
5.90-5.73(m,1H,C-CH,cholesteryl),5.05(m,1H,CH),3.83-3.65(m,1H,OCH,Cholestearyl), 3.60(s,3H,CH_3),2.90(s,2H,NH_2),0.71-1.97(m,43H,CH,cholesteryl).
Mass (HRMS): m/z 458.50$(M+H)^+$.

2.4. Preparation of Cholesterol-Cysteine conjugates
Pale yellow crystals, yield 88 %; m.p. 167-169^{0}C (decomp.); R_f (DCM/ MeOH 7:3): 0.53; FTIR (ATR, υ cm^{-1}): 705,790, 898 (C-H alkene), 1065, 1098 (C-O), 1468 (C=C), 1738(ester,C=O), 2559(SH), 2932(C-H aliphatic), 3273(NH_2).
^{1}H-NMR (400MHz, DMSO-d_6, ppm):
5.84-5.73(m,1H,C-CH,cholesteryl),4.60(m,1H,CH),3.63-3.51(m,1H,OCH,Cholestearyl), 2.82(s,2H,NH_2),2.20(m,2H,CH_2),1.65(t, J=9.2Hz,1H,SH), 0.73-1.91(m,43H,CH,cholesteryl).
Mass (HRMS): m/z 490.75$(M+H)^+$.

2.5. Preparation of Cholesterol-Aspartic acid conjugates
Faint red colored crystals, yield 84%; m.p. 158-160^{0}C (decomp.); R_f (DCM/MeOH 7:3): 0.50; FTIR (ATR,υ cm^{-1}):728,774,863(C-H alkene),1070,1093(C-O),1474(C=C),1749(ester, C=O), 1740(ester, C=O), 2945(C-H aliphatic), 3266(NH_2).
^{1}H-NMR (300MHz, DMSO-d_6, ppm):
5.47-5.35(m, 1H, C-CH, cholesteryl), 4.70-4.55(m, 1H, CH), 3.67-3.54(m,1H,OCH, Cholestearyl), 3.40(q, J=4.5Hz, 2H, CH_2), 3.05(m,2H,CH_2) 2.92(s,2H,NH_2), 0.67-1.81(m,86H, CH, cholesteryl).
Mass(HRMS): m/z 870.39 $(M+H)^+$

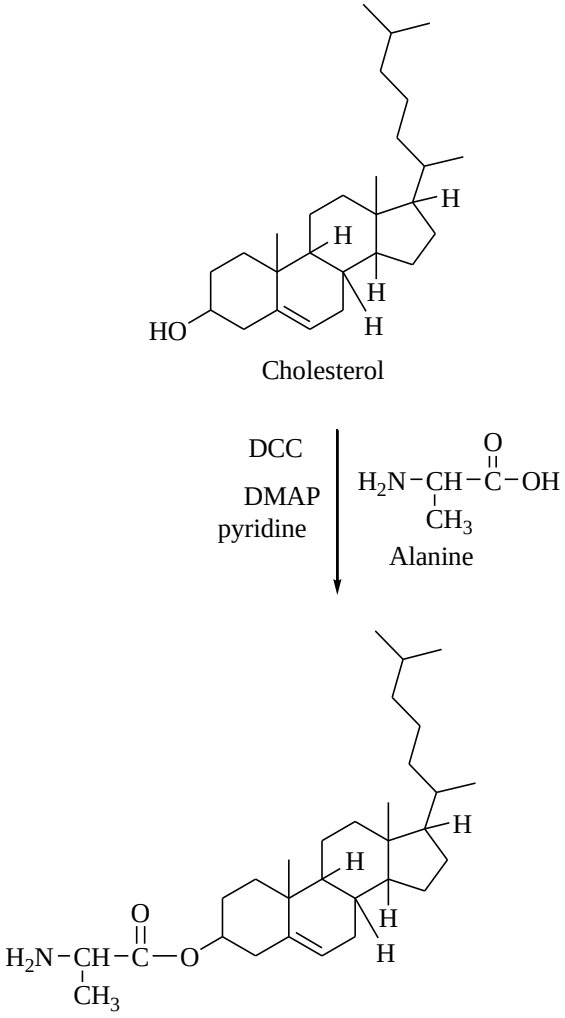
2.6. In-vitro cytotoxicity [12]
MTT assay was used for determination of cell viability against human lung cancer (A549), liver hepatocellular carcinoma (HepG2), Human colon cancer (HT-29), Breast carcinoma (MCF-7), Glioblastoma cell lines (U87 MG) and Normal mouse fibroblast (L929). Cells were seeded in a 96-well plate and incubated for 24 hrs before the assay. Drug conjugates were diluted with the growth medium to give final concentrations of 1000-31.25 µg/ml. Seeded cellular solution of conjugate acts as positive control, was incubated for 24hr at 37^{0}C. Cell solutions without sample were incubated with medium, acts as negative control. Sample solutions were removed from well after 24 hr, MTT dye was added in each well. Solutions were incubated for further 3hr.MTT cellular solution were removed and treated with DMSO for dissolution of formazan crystal. Absorbance of these dye treated cellular solution was determined by Elisa Microplate reader at 570 nm.
Cell viability^{24} = (Absorbance of sample/Absorbance of control) x 100

3. RESULTS AND DISCUSSION

3.1. Chemistry

Biomolecular scaffolds were developed by chemical reaction of L-alanine, L-Cysteine and L-Aspartic acid by conjugation with cholesterol using DCC, DMAP and pyridine base. This esterification reaction type was popularly known as Steglich esterification.

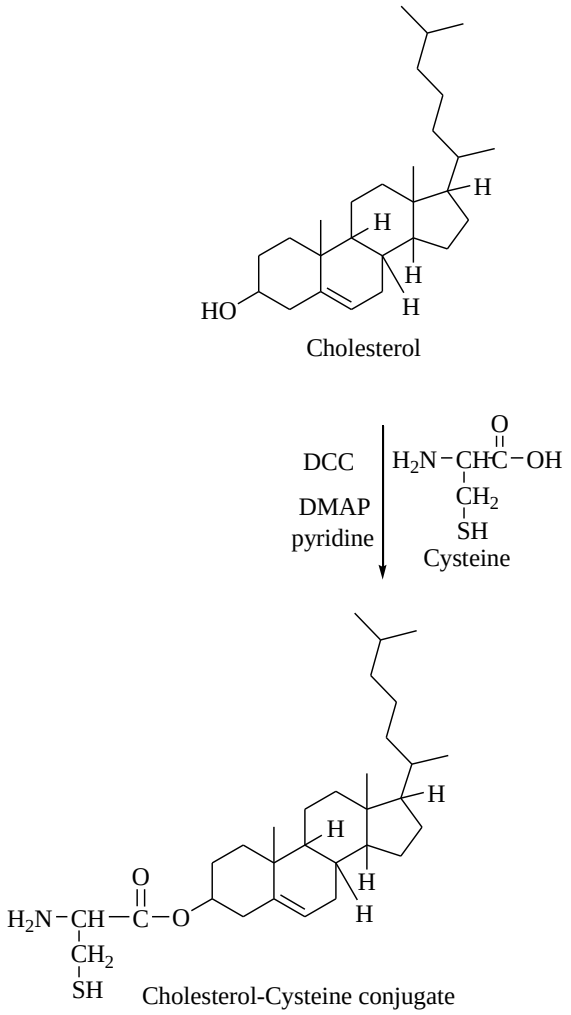
Concerned method deals with formation of O-acylurea intermediate of L-amino acid. Under basic pH conditions anhydride intermediate of amino acid interact with hydroxyl functional group of cholesterol to form ester prodrugs **[**13,14**]**.



Scheme 1. Synthesis of Cholesterol-Alanine ester scaffold.

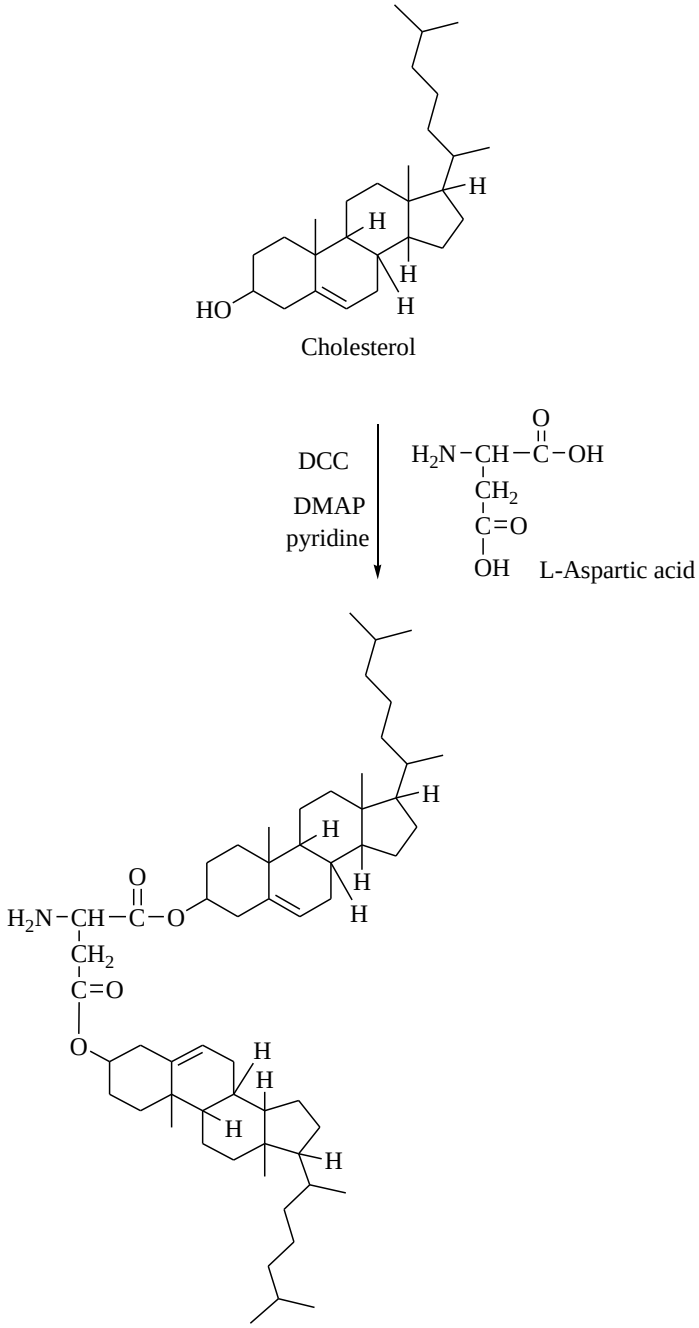
In (Scheme 1) formation of ester functionality was observed in alanine containing scaffold at 1743cm^{-1} in IR spectra.^{1}H NMR of scaffold reflected presence of cholesteryl C-H protons within 0.71-1.97ppm.Linkage of -OCH moiety attached to cholesteryl steroidal nucleus was observed at 3.83-3.65ppm.Multiplet of C-CH cholesteryl was found at 5.90-5.73ppm[5]. Confirmation for presence of methine (C-H) group in motif was highlighted at 5.05ppm.Signal of methyl (CH_3) functional group of alanine was affirmed at 3.60ppm[15]. IR stretching frequency of methyl

group was visualized in spectra at 2940cm^{-1}.Amine group of amino acid was indicated at 2.90ppm.Fragmentation pattern of compound suggested scaffold formation in high resolution mass spectra.



Scheme 2. Synthesis of Cholesterol-Cysteine ester scaffold.

Molecular scaffold formation in (Scheme 2) indicated ester functional group of prodrug at 1738cm^{-1}. Presence of cysteine (SH) moiety in biomolecular entity was seen at 2559cm^{-1} in IR spectra [16]. Proton of C-CH region in cholesteryl was reflected at 5.84-5.73ppm. Presence of methine region(C-H) in cysteine was observed at 4.60ppm.Cholestearyl –OCH functionality was observed at 3.63-3.51ppm. Thiol group functionality was observed at 1.65ppm region in proton NMR spectra [17]. Multiplet of methylene (CH_2) was indicated at 2.20ppm. Mass spectra revealed formation of conjugate in studies.



Scheme 3. Synthesis of Cholesterol-Aspartic acid ester scaffold.

Molecular functionalities in scheme (3) indicated confirmation of ester containing molecular network at 1749cm^{-1}.Presence of lower C-H alkene entity in developed molecule was observed from IR spectra at 728,774,863cm^{-1}. Alkyl group in Aspartic acid was reflected at 2945cm^{-1}. Cholesteryl C-CH functionality was indicated in proton NMR signals at 5.47-5.35ppm. Multiplet of methine (C-H) was indicated at 4.70-4.55ppm.Cross linking of aspartic acid with cholesteryl chain was observed from -OCH proton signals in conjugate at 3.67-3.54ppm. Quartet of methylene(-CH_2) group of aspartic acid was reflected in conjugate at 3.40ppm[18]. Signal of

cholesteryl CH entity was appeared in spectra. Molecular confirmation was done by Mass spectra of bioscaffold.

3.2. Cytotoxicity

Cell viability assay of developed bio moiety indicated potential results for *in-vitro* studies.

Table 1. IC_{50} values of biochemical scaffold in (µM) against cancer cell lines.

Sample Code	Compound Name	IC_{50} value of A 549 [uM]	IC_{50} value of Hep G2 [uM]	IC_{50} value of HT 29 [uM]	IC_{50} value of MCF-7 [uM]	IC_{50} value of U87 MG [uM]
F1	Ala-Cholesterol scaffold	0.46	0.31	0.168	0.16	1.51
F2	Cyst –Cholesterol scaffold	0.71	0.17	0.11	0.34	1.34
F3	Asp-Cholesterol scaffold	0.60	0.67	0.41	0.48	1.01
MTX	Methotrexate	3.1	2.2	0.177	1.65	2.8

Dembitsky et al. reported lipid and their derivatives for cytotoxic activity due to interaction of these molecules with bio membrane [19]. Recent research shows improvement in activity of anticancer drug due to complexation with lipid and amino acid scaffold [20]. All the biomolecular entities effectively show anticancer activity on multiple types of cell lines. These bio macromolecules targets lysozomal enzymes for inhibiting growth of tumor tissues. Conjugates interfere with cellular proteins responsible for producing cytotoxicity. Conjugates exhibited cytotoxic effect at concentration range 1000-31.25 µg/ml. Developed conjugates showed less activity as compared with MTX standard on specified cell line. Biointeractivity with cellular membranes makes these molecules non-toxic on normal cancer cell lines.

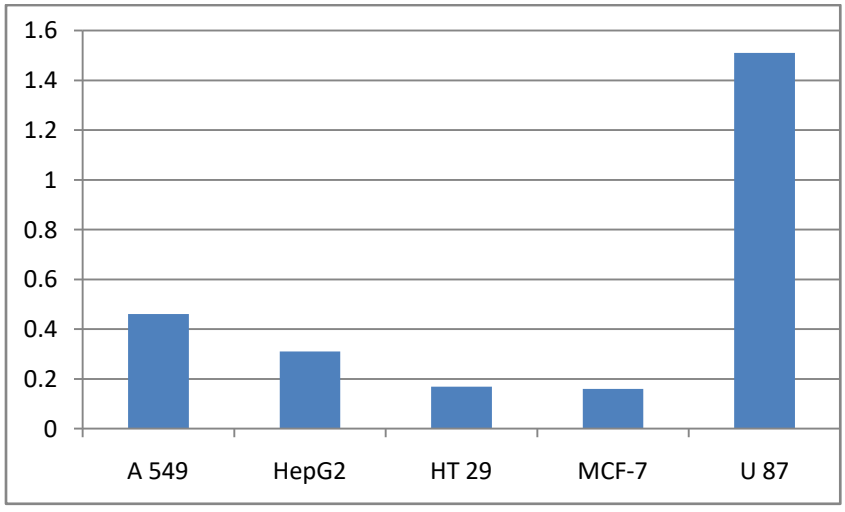


Fig. 1. Alanine Cholesterol scaffold. (IC_{50} values in µM for different cell lines)

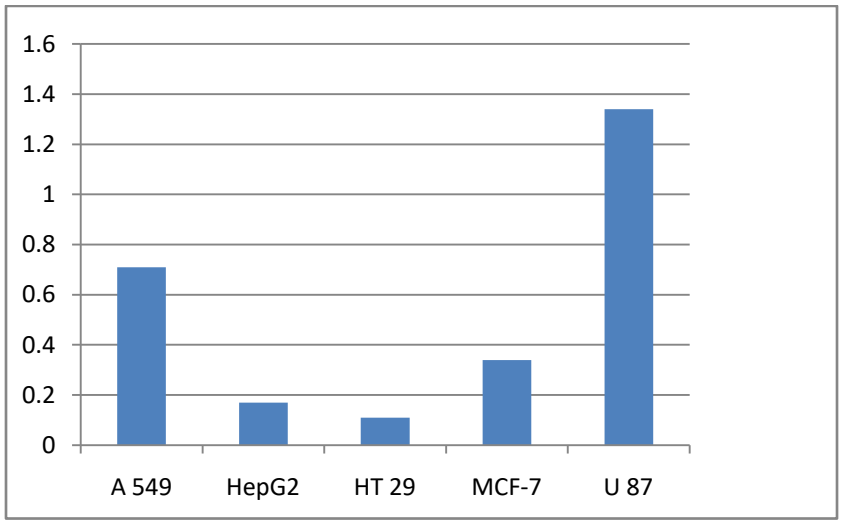


Fig. 2. Cysteine Cholesterol scaffold. (IC_{50} values in μM for different cell lines)

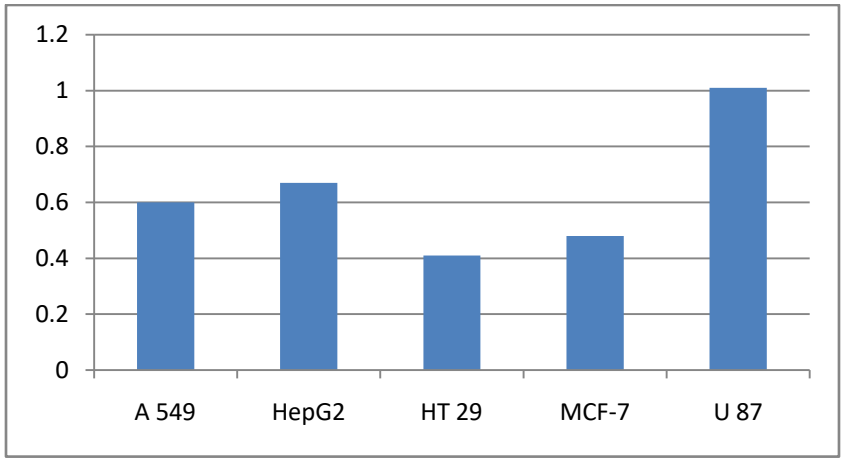


Fig. 3. Aspartic-acid cholesterol scaffold. (IC_{50} values in μM for different cell lines)

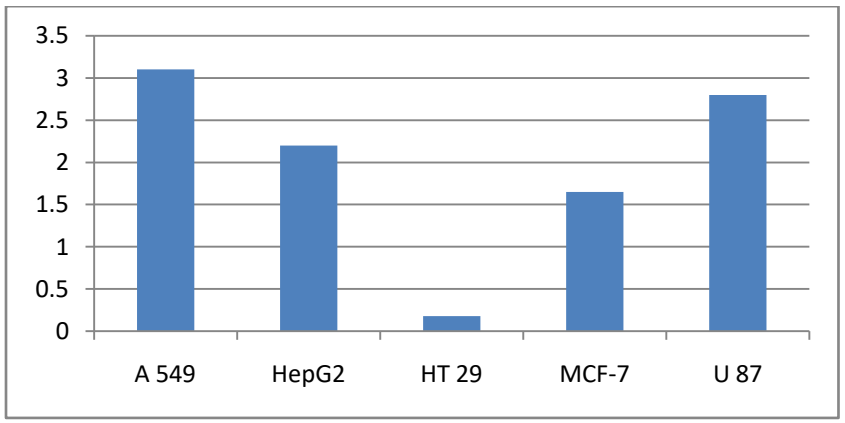


Fig. 4. Methotrexate. (IC_{50} values in μM for different cell lines)

4. CONCLUSION

In the developed method amino acids are linked with lipids using synthetic conjugation strategies. These biological molecules are selective to cancerous cell due their specificity and membrane permeability. Scaffold entities inhibits lysosomal enzyme required for growth of tumor cells. Biomacromolecules reported for increased activity and reduced toxicity by interacting with cellular enzymes. Development of molecular libraries for such type of potent bioactive molecule may lead development in therapeutic drug delivery. Modification in structure of these types of motif may affirm development of potent analog in targeted delivery of anticancer agents.

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