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Synthesis and Characterization of Polymeric Conjugated Sirolimus on MCF-7(Estrogen Positive) and MDA MB 231(Estrogen Negative) Breast Cancer Cell Lines

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ABSTRACT

Sirolimus (SR) is used as an immunosuppressant drug to prevent organ rejection in kidney transplants. It is chemically a macrolide and isolated from the bacterium *Streptomyces hygroscopicus* in a soil sample. Of recent, it has potent antiproliferative properties and useful in the treatment of certain types of cancer. In our objective of the study, SR is chemically conjugated with Methoxy-polyethylene glycolic acid (mPEG COOH) and Poly(lactic-co glycolic acid)[PLGA]. Two polymeric SR conjugates (mPEG-SR and PLGA-SR) were characterized by UV-spectra, infrared (IR) spectra, ^{1}H NMR spectra, matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) and HPLC analyses. It was found to be structurally correlated among the chemical structures of SR, mPEG COOH-SR conjugate, PLGA-SR conjugate, PLGA and m-PEG COOH polymers. The MTT assay of mPEG-SR, PLGA conjugates were carried out on specific MCF-7(Estrogen positive) and MDA MB231 (Estrogen negative) breast cancer cell lines using tamoxifen as control. All results were showing the positive effects of mPEG-SR with IC_{50} values of 15 µg/ml and 1.5 µg/ml more active than tamoxifen with IC_{50} values of 28 µg/ml and 4.9 µg/ml on MDA-MB 231(estrogen negative) and MCF-7(estrogen positive) breast cancer cell lines *in vitro*, respectively. Whereas PLGA-SR conjugate was shown high activity on MCF-7(estrogen positive) with IC_{50} value of 1.2 µg/ml and less activity on MDA MB 231(estrogen negative) with IC_{50} value of more than 100 µg/ml. Both conjugates have not shown any cytotoxicity activity on 3T3 fibroblast normal cell lines. These results indicate that both conjugates may provide highly potent cytotoxicity activity against specific breast cancer types than SR alone. In conclusion, polymeric conjugation is a useful approach in drug delivery systems. These conjugates are basic precursors to formulate into a novel drug delivery system for better release and with increased good bioavailability with fewer side effects.

Key words: Sirolimus, polymeric conjugates, cytotoxicity, breast cancer, MDA-MB 231 and MCF-7 breast cancer cell lines.

INTRODUCTION

Sirolimus(SR) (**1, Fig. 1**) is a macrocyclic compound isolated by *streptomyces hygroscopicus*. It has been widely used as an immunosuppressant drug in kidney transplants.[1,2] In addition to immunosuppressant therapy, sirolimus is used in various types of cancer as a individual drug or in combination.[3] Sirolimus is rapidly absorbed and bioavailability is approximately about 14% after administration of oral solution. However, the absorption has also been decreased with high intake of fat along with this drug. This drug is administered more frequently for every 2 hours in kidney transplant patients to maintain systemic concentration for effective immunosuppressant action due to poor bioavailability and low rate of absorption of this drug [4]. Sirolimus has a solubility of about 2.6 µg/ml in water and low oral bioavailability (14%)[5]. These characteristics have limited clinical applications other than low-dose treatment as an immunosuppressant drug. It is also a potent inhibitor of tumor growth with an IC_{50} value≤50nM against various solid tumors reported. It is chemically known as (3*S*,6*R*,7*E*,9*R*,10*R*,12*R*,14*S*,15*E*,17*E*,19*E*,21*S*,23*S*,

26*R*,27*R*,34a*S*)-9,10,12,13,14,21,22,23,24,25,26,27, 32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1*R*)-2-[(1*S*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl -1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3*H*-pyrido[2,1-c][1,4]-oxaazacyclohentriacontine-1,5,11,28,29 (4*H*,6*H*,31*H*)-pentone.

Polyethylene glycol (PEG) and methoxy polyethylene glycol (mPEG) are linear or branched, neutral polymers available in a variety of molecular weights with low polydispersities (M_w/M_n<1.05). These non-toxic polymers are soluble in water/organic solvent. They have been found useful in biological and pharmaceutical applications especially in conjugation. The conjugation of these polymers with insoluble or sparingly water-soluble drugs is to make more water soluble PEGylated drug conjugates. PEGylation of organic molecules has been reported to enhance aqueous solubility of the organic molecule and to confer other beneficial properties such as improved plasma half-life, improved biological distribution, and reduced toxicity.[6]

The lipase-catalyzed acetylation of sirolimus has been discussed in US Patent Application Publication. This enzymatic process gives sirolimus 4-ester derivatives regiospecifically from sirolimus with excellent yield under mild condition.[6]

The preparation of PEG conjugates of sirolimus or its derivatives has been described in US Patent publications (US Patent Nos 5,955,457; 5,780,462; 6,432,973 and 6,331,547)[7-10].The hydroxyester of sirolimus(CCI-779) was prepared and then the pegylated CCI-779 conjugate was made from, was described in the US Patent publications. (US Patent No. 5,362,718)[11]. These patents describe conjugates formed by chemically linking sirolimus or its derivatives to methoxy polyethylene glycol compounds such as a thiol derivative (mPEGSH) through an ester linkage. Solvent extraction and chromatography purification were thereby required to recover the desired PEG conjugate. Sirolimus 4-iodoacetate was prepared in a 55% yield after high performance liquid chromatography (HPLC) purification.

Sirolimus is conjugated with polyethylene glycol(PEG) to prepare water soluble PEG-sirolimus ester conjugates, which has been claimed and patented under US Patent number(US7605257).[12] The mPEG conjugate is prepared using m-PEGSH conjugated to sirolimus. In one embodiment, the esters and ethers of sirolimus are esters and ethers of the hydroxyl group at the 9-position of the sirolimus nucleus, esters and ethers of a hydroxyl group at the 11-position (following chemical reduction of the 11-ketone), esters and ethers of the hydroxyl group at the 4-position, particularly hydroxyalkyl, hydroxyalkenyl, hydroxyalkylaryl esters or ethers of hydroxyl group at the 4-position of the sirolimus.

The oximes, hydrazones, and hydroxylamines are derived from the ketone of the sirolimus nucleus In other embodiments, 9-esters and ethers of sirolimus are described in the following patents: alkyl esters (U.S. Pat. No. 4,316,885)[13]; aminoalkyl esters (U.S. Pat. No. 4,650,803)[14]; fluorinated esters (U.S. Pat. No. 5,100,883)[15]; amide esters (U.S. Pat. No. 5,118,677)[16]; carbamate esters (U.S. Pat. Nos. 5,118,678)[17]; silyl ethers (U.S. Pat. No. 5,120,842)[18]; aminoesters (U.S. Pat. No. 5,130,307)[19]; acetals (U.S. Pat. No. 5,151,413)[20]; aminodiesters (U.S. Pat. No. 5,162,333)[21]; sulfonate and sulfate esters (U.S. Pat. No. 5,177,203)[22]; esters (U.S. Pat. No. 5,221,670)[23]; alkoxyesters (U.S. Pat. No. 5,233,036)[24]; O-aryl, -alkyl, -alkenyl, and -alkynyl ethers (U.S. Pat. No. 5,258,389)[25] carbonate esters (U.S. Pat. No. 5,260,300)[26]; arylcarbonyl and alkoxycarbonyl carbamates (U.S. Pat. No. 5,262,423)[27]; carbamates (U.S. Pat. No. 5,302,584)[28]; hindered esters (U.S. Pat. No. 5,385,908)[29]; heterocyclic esters (U.S. Pat. No. 5,385,909)[30]; gem-disubstituted esters (U.S. Pat. No. 5,385,910)[31]; amino alkanoic esters (U.S. Pat. No. 5,389,639)[32]; phosphorylcarbamate esters (U.S. Pat. No. 5,391,730)[33]; amino carbamate esters (U.S. Pat. No. 5,463,048)[34]; hindered N-oxide esters (U.S. Pat. No. 5,491,231)[35]; biotin esters (U.S. Pat. No. 5,504,091)[36]; and O-alkyl ethers (U.S. Pat. No. 5,665,772)[37]. The preparation of these esters and ethers is described in the patents listed above.

The above esters, ethers and other PEGylated conjugates, none of them are of marketing interest and further proceedings have not been reported in terms of their applicability and advantage in terms of their use as precursors for drug delivery systems. Most of the studies are surrounded to improve immunosuppressant action rather than exploring cytotoxicity activity of these conjugates and their chemistry at macromolecular and cellular environment.

Paolo Baldo et al, reported potential usefulness of sirolimus (**1**) in cancer chemotherapy and especially in human breast cancer. It has been evident that sirolimus (**1)** was used as an mTOR (mammalian Target of Rapamycin) inhibitor and also involved in mTOR pathway. They have concluded the components of mTOR pathway with evidence of

involvement in human breast cancer.[38] Sirolimus (**1**) was proven as more effective in the inhibition of pre-malignant lesions than in reducing invasive cancer especially in breast cancer cell lines. A notable effect of sirolimus (**1**) treatment was on cell proliferation in cancer areas that have high replication in breast cancer cell line types. [39] This molecule of our interest, we have aimed to synthesize the polymeric conjugates proceeding further with direct and simpler conjugation process. In the present study, we developed the synthetic process for the preparation of PEGylated sirolimus **2**(figure 2) and poly(lactic-co-glycolic acid) conjugated sirolimus **3**(figure 2) via Steglich esterification by using dicyclohexyl carbodiimide (DCC) and 4-dimethylamino pyridine (DMAP) as a catalyst.[40] This coupling reagent is widely used to get a high yielding ester formation between acidic group of the polymer and hydroxyl group(s) of sirolimus (**1**). These conjugates **2** and **3** were formed by an ester linkage on the macrocyclic structure bearing OH groups in the positions 4 and 9 on the skeleton of sirolimus (**1**). These conjugates **2** and **3** were screened for antiproliferative activity against two human breast cancer cell lines MCF-7(Estrogen Positive) and MDA MB 231(Estrogen Negative) and it has also been studied cytotoxicity on 3T3 fibroblast cells (normal cells) to prove no toxicity of the conjugate on normal cells. The expected hypothesis of these conjugates **2** and **3** are more active than when correlated with sirolimus, **1** alone.

EXPERIMENTAL SECTION

General experimental conditions: All reactions were carried out in oven-dried glassware. All starting materials were purchased from Sigma-Aldrich, while the solvents used were procured of Spectroscopic and chromatographic grade. Solvent mixtures employed in chromatography were reported as volume to volume ratios. Melting points were determined on a Stuart apparatus (SMP3) and are uncorrected. IR spectra were recorded on a Thermo electron corporation Perkin Elmer FT-IR spectrophotometer using KBr disc method. 1H NMR and 13C NMR spectra were recorded at 500 MHz on Bruker DRX-600 spectrometer in the dimethyl sulfoxide (DMSO-d6) solvent. Chemical shifts (d) for proton and carbon resonances are quoted in parts per million (ppm) for **1**, **2** and **3.**

General procedure for the synthesis of methoxy poly(ethylene glycolic acid)

Synthesis of methoxy-Poly(ethylene glycolic acid) [m-PEG-COOH](P_1): The methoxy polyethylene glycol (MPEG- 2000) (0.175 moles) was prepared accurately and transferred into a round bottom flask. 0.931 moles of sodium carbonate (Na_2CO_3) and 0.323 moles of potassium permanganate ($KMnO_4$) were prepared separately in purified water. Both solutions were mixed and added to the round bottom flask containing MPEG-2000. The mixture was stirred vigorously on magnetic stirrer for 3-4 hours at 4-5°C by immersing in an ice bath. The reaction mixture was allowed to reach to the room temperature. The precipitated manganese dioxide was removed by filtration. The filtrate is obtained, cooled and heated continuously to get a concentrate filtrate of about 100 ml. The solution of filtrate was cooled and covered with a layer of ether. The solution was kept aside for the separation of ether and aqueous layer. The extraction of aqueous layer was done by two or three portions of ether. The collected aqueous layer was heated on a water bath for removal of ether. The precipitated methoxy polyethylene glycolic acid (mPEG COOH) (**2**) was filtered.[42]

General procedure for the synthesis of conjugate 2

Synthesis of methoxy PEGylated sirolimus (2): The methoxy poly(ethylene glycolic acid) (**P_1**)[0.2%] in a 500-mL of single-necked flask in 200 mL of dichloromethane(DCM) and 4.9 mmol of sirolimus(**1**) and 0.03 moles of 4-dimethylaminopyridine were added. The solution was stirred and cooled in an ice bath to 0°C, while 0.03 moles of dicyclohexylcarbodiimide was added over a 5-minutes period. The reaction mixture was stirred for 24 hrs at room temperature. The organic solution was dried over anhydrous sodium sulfate and the organic solution is concentrated with a rotary evaporator and DCM was removed by using rotary evaporator under reduced pressure. The PEGylated sirolimus conjugate (**2**) was purified using by gel permeation chromatography using Sephadex G-15 and kept in a refrigerator for a week. The freeze dried sample was stored in a sealed container.

The conjugation was confirmed by IR (KBr disc method) at ν:1735 cm^{-1}(The carbonyl stretch C=O of aliphatic esters); ν:1075 cm^{-1}(C–O stretch);ν:2975 cm^{-1}(C-H Stretch, alkyl);^{1}H NMR (500 MHz, DMSO-*d*6) ppm 0.74 - 0.84 (m, 1 H) 0.92 - 0.97 (m, 1 H) 0.98 - 1.04 (m, 1 H) 1.07 - 1.14 (m, 1 H) 1.16 - 1.26 (m, 2 H) 1.45 -1.51 (m, 1 H) 1.55 - 1.61 (m, 1 H) 1.63 - 1.73 (m, 2 H) 2.43 - 2.53 (m, 1 H) 3.12 - 3.18 (m, 1 H) 3.20 - 3.23 (m, 1 H) 3.25 - 3.35 (m, 2 H) 3.37 - 3.41(m, 1 H) 3.43 - 3.52 (m, 11 H) 3.48 (s, 24 H); ^{13}CNMR (126 MHz, DMSO-*d*6) ppm 33.80 (s, 1 C) 39.45 (s, 1 C) 39.62 (s, 1 C) 39.79 (s, 1 C) 39.95 (s, 1 C) 40.12 (s, 1 C) 40.21 (s, 1 C) 40.29(s, 1 C) 40.46 (s, 1 C) 70.22 (s, 1 C);

General procedure for the synthesis of conjugate 3 Synthesis of PLGA conjugated sirolimus (3): A 500-mL of one-necked flask equipped with a calcium chloride drying tube was filled with a solution of 1.32% of methoxy poly(Lactic-co-glycolic acid) [**PLGA, P_2**] in 200 mL of dichloromethane (DCM) and 1.0 mmol of sirolimus and 0.60 mmoles of 4-dimethylaminopyridine(DMAP) were mixed with stirring. The solution was cooled in an ice bath to 0°C, while 0.60mmoles of dicyclohexylcarbodiimide(DCC) is added over a 5-minutes period. After the reaction mixture was stirred for 24 hrs at room temperature. The organic solution was dried over anhydrous sodium sulfate and the organic solution is concentrated and DCM was removed under reduced pressure by using rotary evaporator. The PLGA sirolimus conjugate (**3**) was sealed and kept in a refrigerator for a week. The freeze dried sample was stored in a sealed container. The conjugate was confirmed by IR(KBr Method) at ν:1780cm^{-1}(The carbonyl stretch C=O of aliphatic esters); ν:1100 cm^{-1}(C–O stretch);ν:2975 cm^{-1}(C-H Stretch, alkyl);^{1}H NMR (500 MHz, DMSO-*d*6) ppm 1.18 - 1.28 (m, 1 H) 1.18 - 1.28 (m, 1 H) 1.38 - 1.48 (m, 24 H) 2.42 - 2.52 (m, 2 H) 3.30 (br. s., 2 H) 4.82 - 4.90 (m, 13 H) 5.14 - 5.23 (m, 6 H) 5.73 (s, 1 H); ^{13}C NMR (126 MHz, DMSO-*d*6) d ppm 16.89 (s, 1 C) 16.90 (s, 1 C) 16.96 (s, 1 C) 17.05 (s, 1 C) 17.05 (s, 1 C) 39.41 (s, 1 C) 39.46 (s, 1 C) 39.52(s, 1 C) 39.58 (s, 1 C) 39.63 (s, 1 C) 39.74 (s, 1 C) 39.80 (s, 1 C) 39.96 (s, 1 C) 40.05 (s, 1 C) 40.13 (s, 1 C) 40.22 (s, 1 C) 40.30 (s, 1 C) 40.39 (s, 1C) 40.46 (s, 1 C) 40.56 (s, 1 C) 40.56 (s, 1 C) 55.35 (s, 1 C) 61.16 (s, 1 C) 69.16 (s, 1 C) 69.19 (s, 1 C) 69.22 (s, 1 C) 69.26 (s, 1 C) 167.03 (s, 1 C)167.14 (s, 1 C) 169.68 (s, 1 C).

MTT cytotoxicity Assay: Cell culture with conc. of 1 X 10^5 cells/ml was prepared and plated onto (100µl/well) 96-well plates and after the cells reached 50–60%confluence, were treated with 1.56-100µg/ml of conjugates **2**, **3** and reference compound **1** and Tamoxifen (control). After 72 h the percentage of cell viability was determined. Data are plotted as % to one of the arbitrary selected compounds of three independent experiments performed in duplicates.[43]

RESULTS AND DISCUSSION

Chemistry: The synthesis of polymeric drug conjugate esters **2, 3** is outlined in Scheme 1 and Scheme 2. Specifically, 4-dimethylamino pyridine (**4**) was reacted with methoxy-poly(ethylene glycolic acid) (**m-PEG-COOH**, **P_1)** or poly(lactic-co-glycolic acid(**PLGA, P_2**) to produce unstable polymeric carboxylate ion (**5**). By the reaction of **5** with dicylcohexylcarbodiimide (**6**), the coupling agent (**7**) is formed, which was conjugated with sirolimus(**1**) to give moderate to good yields of m-PEGylated sirolimus (**2**) and PLGA-sirolimus conjugate (**3**) via Steglich esterification. In this process, the esterification approach has been completed efficiently with fewer efforts and achieved better yields of both conjugates **2(**80-90%) and **3**(90-95%). Both conjugates were purified and confirmed for the ester functional group by FT-IR spectroscopy. The attachment of carboxy group of both polymers was shown at 4R and 9R positions in the figure 2. The hydroxy group at 27- position could not seem to be participated in the reaction due to steric hindrance of 26R methyl group and stable conformation at that position under these conditions. The carbonyl group at 28-position is highly electrophilic due to the electronegativity of oxygen atom. The nucleophilicity of carbonyl group increases to facilitate hydrogen bonding with hydrogen on 27R hydroxyl group. The stereochemistry of both conjugates **2** and **3** has not been affected and retained the original configuration of the sirolimus molecule at positions 4R and 9R.

Antiproliferative activity: The solubility of two conjugates **2** and **3** has been evaluated by software and also theoretically by c log P[41] . They were compared to sirolimus (Table I). The theoretical calculations for conjugates **2** and **3** were shown and the values are generally lower than that of compound **1** with reduced lipophilicity.

These two conjugates were characterized by *in vitro* MTT assay performed on two human breast cancer cell lines [MCF-7(estrogen positive) and MDA-MB 231(estrogen-negative)] and 3T3 fibroblast cells using tamoxifen as reference compound. The results were expressed as IC_{50} values as shown in Table I.

The results were obtained and highlighted for the compound **1** and conjugates **2** and **3** for its antiproliferative activity comparable or even higher with respect to that of tamoxifen (reference drug 1) with regards to the estrogen positive MCF-7 cells. In particular, the most active compound conjugate **3** had an antiproliferative activity about four times higher than the reference drug. The conjugate **2** had more than three times an antiproliferative activity to that of tamoxifen as well. Whereas the compound **1** has shown significant activity more than two times to that of reference drug.(figure 3).

In corresponding to the estrogen negative MDA-MB231 cells, the results are correlated with the compound **1** and conjugates **2** and **3** for its antiproliferative activity to that of tamoxifen (control). In particular, the most active compound is **2** that showed an antiproliferative activity about two

times higher than the reference drug (figure 4).The conjugate **3** and the compound **1** were not shown any significant activity than that of the reference drug. The study on 3T3 fibroblast cells (normal cell lines) of the compound **1**, conjugate **2** and conjugate **3** has shown nontoxic to normal cells (figure 5).

CONCLUSIONS

The synthesis through Steglich esterification reaction of carboxylic acid bearing polymers with hydroxyl bearing sirolimus are clinically used to treat breast cancer, is described. The investigation of the antiproliferative effect on human breast cancer cell line MCF-7 showed for both conjugates **2** and **3** an interesting cytotoxicity which is significantly higher with respect to that obtained for tamoxifen. These results could be also in agreement with the difference in cytotoxicity between **1** and its conjugates **2** and **3** to exert better antitumor effect on human breast cancer. Both novel sirolimus conjugates **2** and **3**, it is appeared that induces the highest antiproliferative effect on MCF-7 cells, about four times higher with respect to that of tamoxifen. In addition, MDA MB 231cell lines show a significant less sensitivity toward **1** and conjugates **2** and **3**. These conjugates with improved physicochemical properties reduced particle size and increased bioavailability rendered as nano drug-carriers to obtain more drug release at specific for the treatment of breast cancer types than conventional drug preparations.

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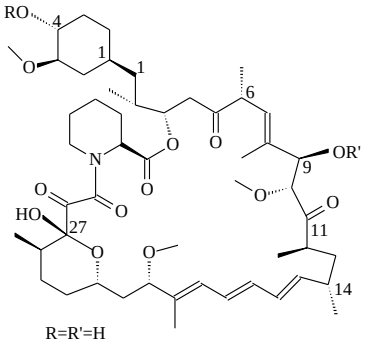

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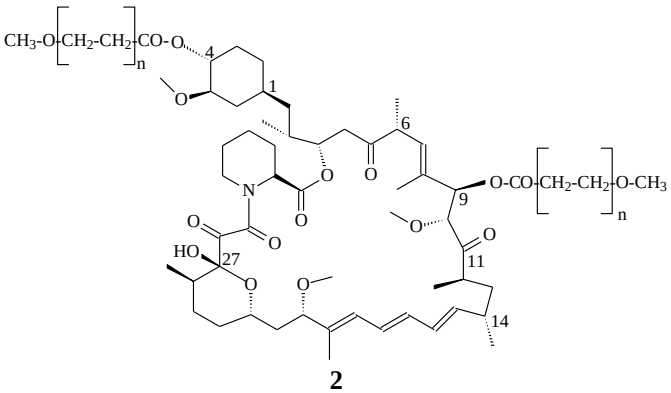
Figure 1. Structure of sirolimus (**1**)



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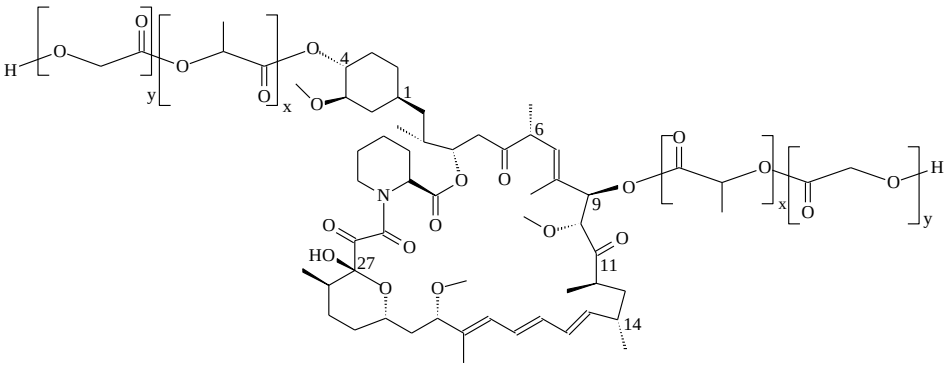

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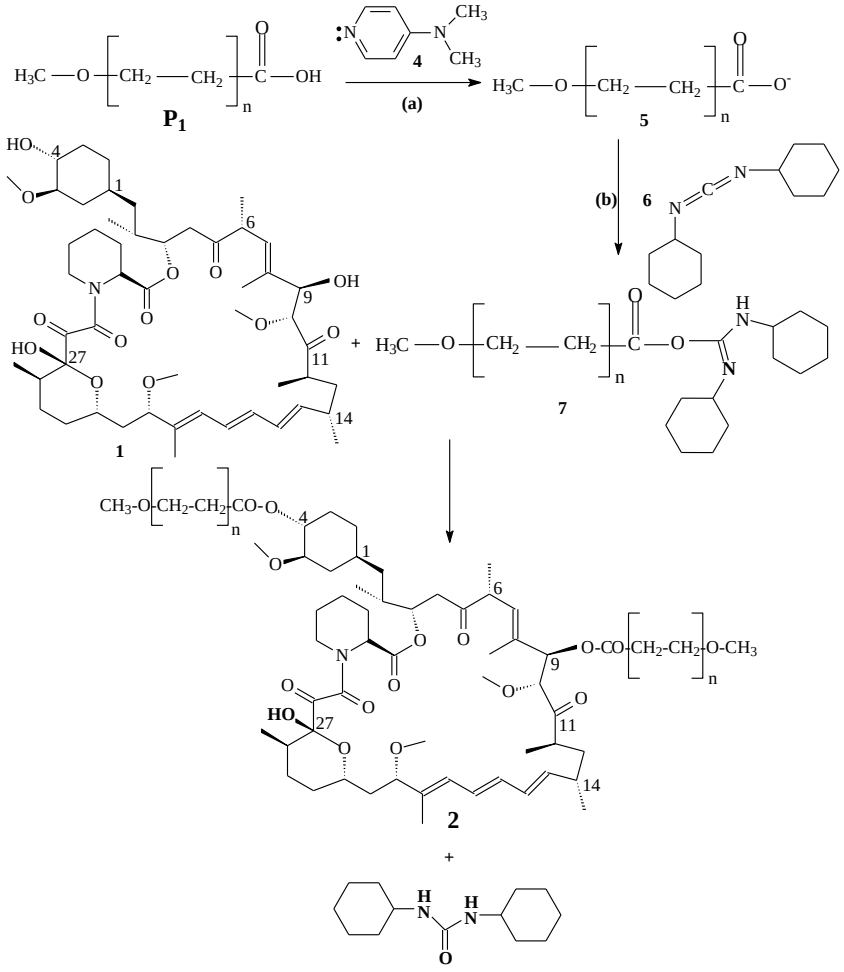
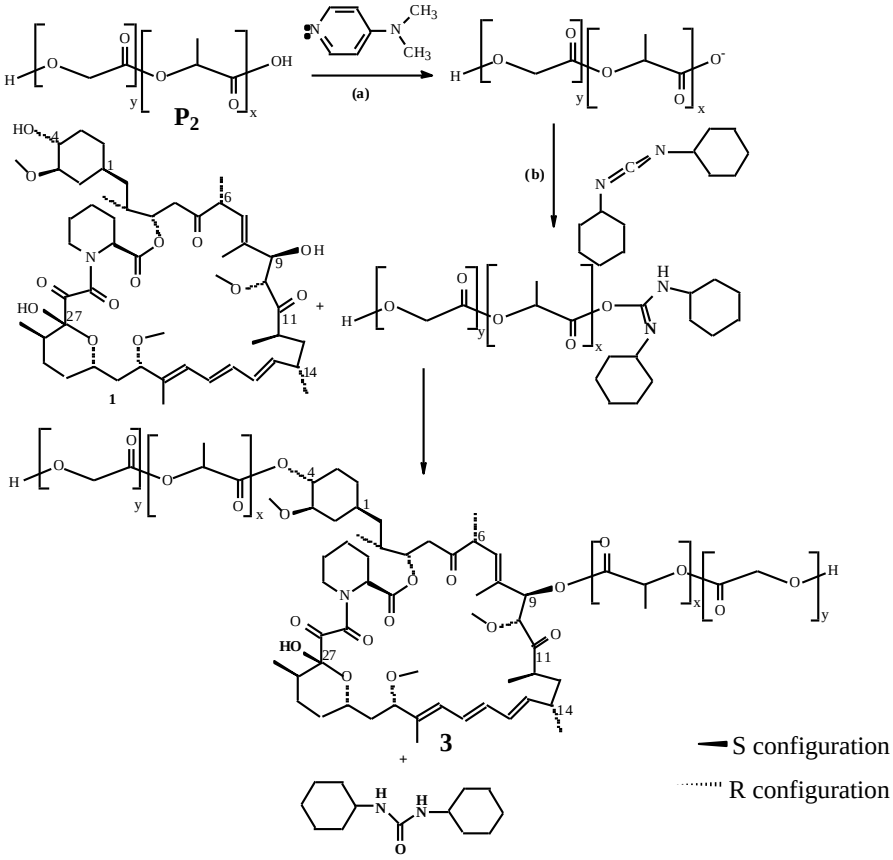
Figure 2. Expected structure of m-Poly (ethylene glycolic acid) [mPEGCOOH] ester of sirolimus (**mPEGylated sirolimus conjugate) (2);** Expected structure of Poly (lactic-co-glycolic acid) [PLGA] ester of sirolimus (**PLGA-sirolimus conjugate) (3);**



Scheme 1. Reagents and conditions: (a) DCM, 0°C; (b) RT, 24 hrs;



Scheme 2. Reagents and conditions: (a) DCM, 0°C; (b) RT, 24 hrs;

Table I: Cell growth inhibition in presence of conjugates and reference compounds

Compound	Cell lines[a](IC$_{50}$ values in µg/ml)			cLog P[b]
	MCF-7	MDA MB 231	3T3	
1	1.8	>100	no cytotoxicity	4.917
2	1.5	15	no cytotoxicity	1.397
3	1.2	>100	no cytotoxicity	2.221
Tamoxifen	4.9	28	no cytotoxicity	6.064

[a] values are the mean±SD of at least three independent experiments
[b] cLog P is calculated octanol-water coefficient

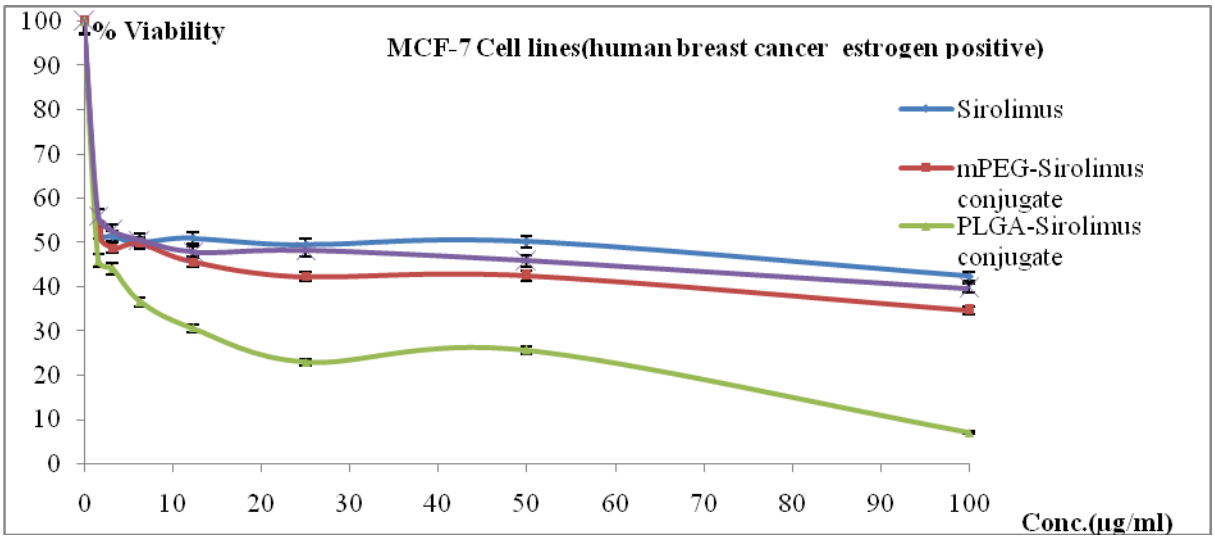


Figure 3: Cytotoxicity of compound **1**, conjugate 2, conjugate **3,** and **tamoxifen** on MCF-7 cell lines.

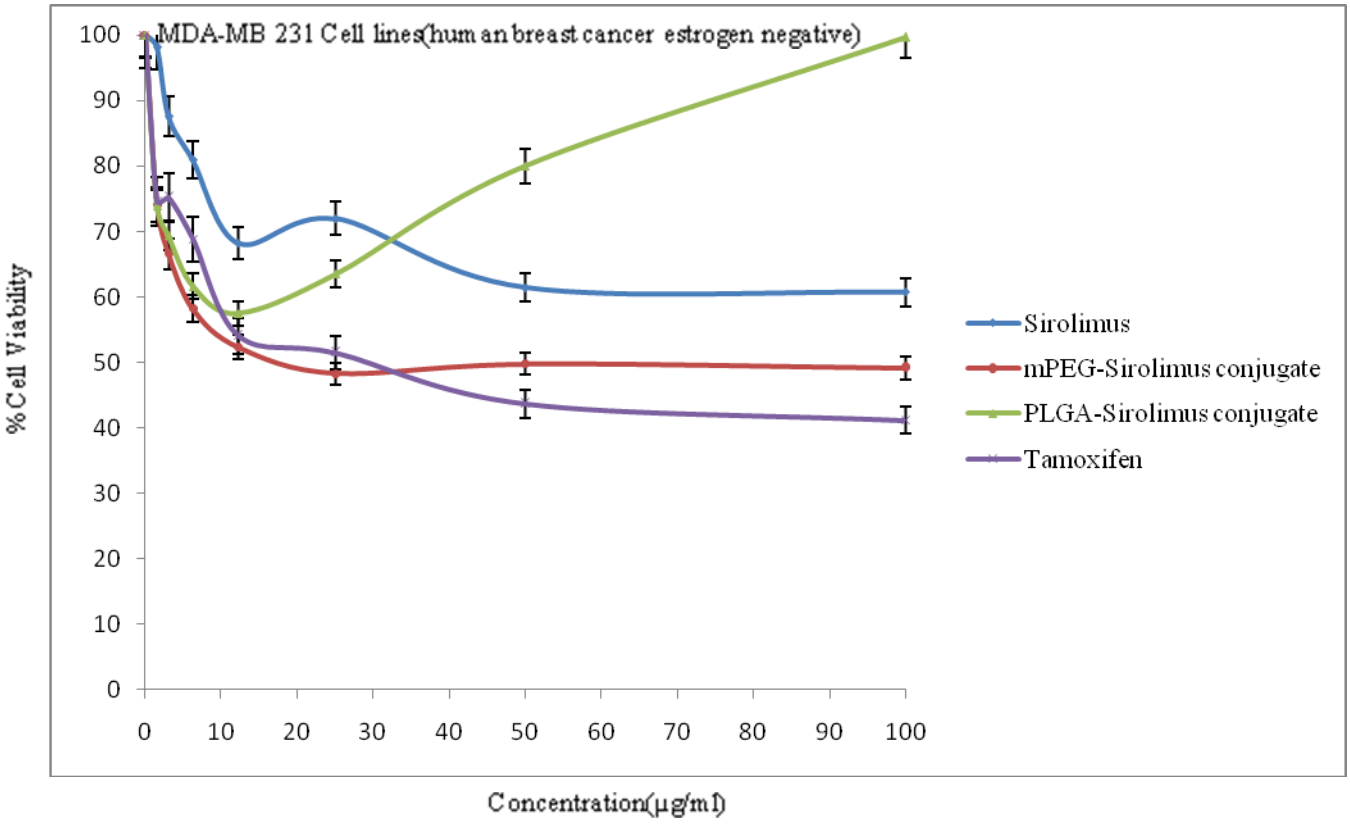


Figure 4. Cytotoxicity of compound **1**, conjugate 2, conjugate **3,** and **tamoxifen** on MDA MB231 cell lines.

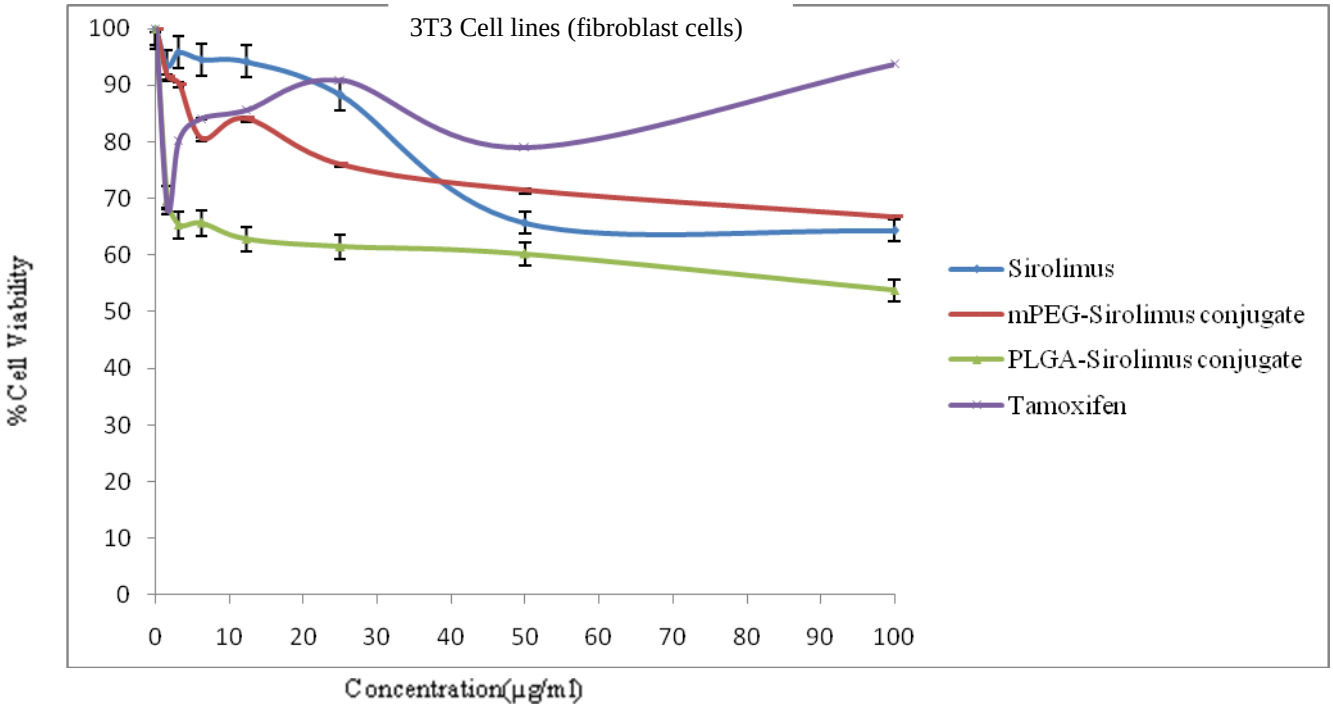


Figure 5. Cytotoxicity of compound **1**, conjugate **2**, conjugate **3,** and **tamoxifen** on 3T3 fibroblast cells.

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