ENGINEERED ATENOLOL-GLYCOCONJUGATES TO TARGET H9C2 CARDIOMYOCYTE CELL LINES

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Abstract
Background: One of the most important fields of biomedical engineering study nowadays is targeted drug delivery to specific cells. A drug's therapeutic efficacy can be improved and optimised by tightly targeting it to a pathophysiologically essential tissue architecture. The goal of this research is to develop saccharide conjugates for the targeted delivery of Atenolol, a -blocker.
Methods: Galactose (monosaccharide), pectin (polysaccharide), and chitosan were chosen as the saccharides (polysaccharide). By grafting Atenolol with the modified saccharides, the conjugates were created. Spectroscopic and thermal studies were used to describe the chemically changed saccharides conjugates. H9c2 cell lines were used to conduct drug release research and cellular uptake studies. To investigate cytotoxicity, a brine shrimp lethality test was done.
Results: The outcomes exhibit that Atenolol-modified saccharide conjugates can productively convey the medication to the target.
Conclusion: It can be inferred that the improvement of saccharide-drug conjugates can be a compelling methodology for targeting cardiovascular medication.

Keywords
glycoconjugates; atenolol; targeting

Introduction

The capacity to target a medicine to specific cells can boost its therapeutic efficacy dramatically. Adequate drug dosages delivered to specific areas increase therapeutic outcomes wherever they are needed and hence reduce side effects, potentially resulting in a large reduction in side effects [1–3]. The drug targeting concept, according to Martinez, is frequently related with the utilisation of carrier systems, which can possibly deliver medicines, imaging agents, or therapeutic genes selectively to the site of action.

Natural-source oligosaccharide and polysaccharide polymers are non-toxic, biocompatible, and biodegradable. Other biopolymers, such as lipids and proteins, are less thermally stable than polysaccharides [4,5]. According to Sabyasachi [6] integrating the therapeutic agent within a chemically modified polymeric matrix may help to protect the physiologically active component from degradation, improve absorption, control drug release, improve therapeutic efficacy, and reduce administration frequency. Chemical grafting is a method of connecting one or more species of blocks to the main chain as a side chain, resulting in macromolecular copolymers with different

physicochemical features. The number, length, and molecular structure of the side chains identify the newly created copolymer [7].

Cardiovascular illnesses such as arrhythmia and hypertension are serious public health concerns since they affect a vast number of people around the world. Because of their affinity for sympathetic receptors found in many organs, cardiovascular medicines such as sympathetic antagonists have a variety of side effects. As a result, medications like -blockers must be targeted for cardio-selective activity. Passive or active targeting can be used to deliver these medications to cardiac tissues with precision [8].
Because of anatomical and physiological variations, drug administration to the cardiovascular system differs from drug delivery to other systems [2]. Carbohydrate transporters like GLUT4 (Human Solute carrier family-2) are abundant in cardiac cells and are responsible for glucose transport across the cell membrane. The conjugation of cardiovascular medicines with oligosaccharides will be an appealing strategy for manipulating medication pharmacokinetics [9].
The goal of this project is to alter saccharides and construct saccharide conjugates for targeted administration of Atenolol, the -blockers, in order to improve therapy efficacy while reducing side effects.

Materials and Methods

Materials.

- Chemicals

Wockhardt Limited, Aurangabad, donated the drug atenolol (ATN). Loba Chemicals, Mumbai, offered galactose, pectin, and chitosan. Molychem, Mumbai, provided oxalic acid and thionyl chloride. H9c2 adherent rat heart cells were obtained from the National Center for Cell Sciences (NCCS, Pune, India). Himedia lab provided Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, antibiotics (streptomycin-penicillin solution), foetal bovine serum (FBS), Trypsin-EDTA, Phosphate buffered saline solution (PBS), Hank's Balanced Salt Solution (HBSS), Tris-base, and Triton-X 100, and Qualigens supplied methanol (HPLC grade). Merck Pvt Ltd provided the silica gel aluminium plates 60F254. HPLC column (4.6 mm x 150 mm, 5 m ODS-3, 100 mL) Phenomenex provided the ProdigyTM C18. Tissue culture flask (75 cm^2) with 96 well polystyrene tissue culture plate with flat bottomwere purchased from Tarsons Pvt. Ltd.

- Instruments

Spectrophotometric examination was performed utilising a matched pair of 10-mm quartz cells on a Systronics 2201, India UV-Visible spectrophotometer with a spectral bandwidth of 2 nm and wavelength precision of 0.3 nm. Nicolet-iS10, USA FTIR was used for the FT-IR investigation. On a Differential Scanning Calorimeter-Mettler-Toledo, Switzerland, thermal behaviours were investigated using a nitrogen flow rate of 40 ml/min and a heating rate of 10°C/min from 25 to 300°C. The NMR spectrometer used for the conjugate analysis was a Bruker AV III 400 MHz from Switzerland. The Central Instrumentation Facility, Punyashlok Ahilyadevi Holkar Solapur University, Solapur, Maharashtra, conducted FTIR, DSC, 1H NMR, and 13C NMR research. HPLC analysis was performed on a Jasco MD-2010 multiwavelength detector from Japan, which was equipped at Bharati Vidyapeeth College of Pharmacy in Kolhapur, Maharashtra, Borwin® Version 1.5 software was used. Biocyte Institute of Research and Development in Sangli, Maharashtra, did the cell line investigation.

Synthesis of the Atenolol conjugates by cross-linking the modified saccharides.

The goal of this study was to chemically change the saccharides and conjugate Atenolol for cardiovascular targeting. Using oleoyl chloride, a monosaccharide, galactose (G), and two polysaccharides, pectin (P) and chitosan (C), were chemically changed to produce esters, Galactose oleate (G1), Pectin oleate (P1), and Chitosan oleate (C1). In a three-step approach, chemical modification of saccharides was followed by conjugation of Atenolol with the changed saccharide. AG1, AP1, and AC1 were assigned to the various conjugates.
Step 1: Modified saccharides synthesis (MS).
For the alteration of the Saccharide, the Schotten-Baumann reaction [10] was used.
Over the course of 2 hours, 10 ml of acid chloride in ethanol (20% w/v) was gradually added to the ethanolic solution while stirring. The reaction product was collected, washed, and dried in a hot air oven at 37°C.
Step 2: ATN-MS conjugates synthesis.

The method used to synthesise drug-saccharide conjugates was somewhat modified from that described previously [11]. Over the course of two hours, 10 ml of acid chloride in ethanol (20% w/v) was gradually added to an ethanolic solution of 2 g saccharide and 1 g medicine, stirring constantly. The reaction result was collected, cleaned, and dried at 37°C in a hot air oven.

Physicochemical characterization of MS.

Melting point, partition coefficient, swelling factor, and ester value were used to characterise chemically changed saccharides. TLC, FTIR, and DSC studies were used to confirm the modification reaction.

Characterization of ATN-MS conjugates.

Physicochemical characteristics of the produced conjugates were studied. Melting point and TLC were used as key parameters, followed by FTIR, NMR, and DSC investigation on equipment listed in the Materials section to confirm the reaction.

Drug release analysis

- H9c2 cells preparation

H9c2 adherent rat heart cells were cultured in DMEM with 20 M L-glutamine, 0.45 percent glucose, and 10% v/v heat-inactivated FBS. To prevent microbiological contamination during maintenance in a humidified CO2 incubator, gentamicin sulphate (50 g/ml), penicillin (100 IU/ml), streptomycin (10 g/ml), and amphotericin B (25 ng/ml) were introduced (New Brunswick, Eppendorf). To eliminate any remaining media, cells in a confluent layer were rinsed with 10 ml PBS (pH 7.4). To obtain a homogeneous solution, 0.25 percent w/v Trypsin-EDTA was added to detach the cells from confluency. The cells were mixed in a 1:1 ratio with 0.4 percent w/v Trypan blue solution and counted using a hemocytometer to yield 10^4 cells.

- Cellular uptake study

The above H9c2 cells were cultivated and incubated in a humidified incubator to attain 2×10^5 cells for the cellular uptake investigation [12–14]. The cells were rinsed with 200L HBSS and incubated for equilibrium after incubation. In each well, atenolol-saccharide conjugates (50 g/ml), Atenolol (100 g/ml), and sterile water were added, and they were incubated for 12 hours. The cells were then removed, rinsed in ice-cold PBS (pH 7.4), and incubated for 30 minutes with 0.5 percent v/v Triton-X 100. To prepare samples for HPLC analysis, cells were filled with a 90:10 methanol:water mobile phase and centrifuged (REMI, Mumbai, India). The supernatant layer was diluted and spiked for HPLC analysis to evaluate cellular absorption in H9c2 rat heart cells after centrifugation.

- Chromatographic conditions

The concentration of atenolol in H9c2 rat cardiac cells was determined using the RP-HPLC technique. For chromatographic analysis and data gathering, an HPLC system (Jasco MD-2010, multiwavelength detector) with Borwin Version 1.5 software, Prodigy™ C18 column as stationary phase, and methanol: water (90:10) as mobile phase was employed. A flow rate of 1 ml/min was set, and 20 l samples were injected. The peak's intensity was recorded at 223 nm.

- Toxicity analysis of conjugates

To assess the cytotoxicity of produced conjugates, a brine shrimp lethality test was performed. Brine shrimps (Artemia salina) were hatched in sterile artificial seawater (made with sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) in a 1L conical vessel under continual aeration for 48 hours. After hatching, active nauplii free of eggshells were removed and used for the assay in a brighter part of the hatching chamber. Each vial containing 4.5 ml of brine solution contained ten nauplii drawn through a glass capillary. As a control group, a group of nauplii was left untreated. In the test group, 0.5 ml of test samples (10, 50, 150 g/ml) were mixed with 4.5 ml of brine solution and kept at room temperature for 24 hours under light, with surviving larvae counted [15–17]. The institutional animal ethics council does not have to approve the experimental model chosen for toxicity investigations.

- Determination of lethal dose

By comparing the mean surviving larvae of the test and control tubes, the % lethality was calculated. The best-fit line plotting concentration versus percentage lethality yielded LC50 values. To determine LC50 values, the data were analysed using a Finney computer programme (Probit analysis).

Results and Discussion

The method published by the Schotten Baumann reaction was used to modify saccharides using acid chloride. As a result, galactose oleate, pectin oleate, and chitosan oleate, three distinct modified sachharides saccharides, were produced.

Characterization of modified saccharides (MS).

Characteristics of modified saccharides are depicted in Table 1.

Table 1.Characteristics of synthesized MS**.**

Code	% Yield	Melting point (˚C)	Partition Coefficient	Swelling % ± SD	Ester value	R_f
G	-	178-180	1.28	124±0.44	-	0.2
G1	78.21	153-155	4.74	114±4.97	61.71	0.63
P	-	166-168	2.2	420±3.23	-	0.14
P1	92.60	146-148	6.45	316±0.84	282.97	0.48
C	-	89-91	1.95	204±2.29	-	0.25
C1	72.33	73-78	5.88	186±4.65	206.57	0.55

Characterization of ATN-MS conjugates.

The synthesized conjugates were characterized for various physicochemical parameters, including percentage yield, melting point, and Rf value by thin-layer chromatography followed by FTIR, DSC, 1H NMR, and 13C NMR analysis on instruments as per specified in the Materials section. Confirmation of synthesized compounds was done by the results of the analysis.

Conjugate AG1: Color: colorless, yield: 85%, m.p.: 155-160°C, Rf: 0.74, IR: aldehyde at 1709 cm−1, NH (amide) at 2923 cm^{-1}, NH (amine) at 3288 cm^{-1}, OH at 3384 cm^{-1}, CO (aromatic) 1247 cm^{-1}, DSC: Tg 159°C, NMR: 1H NMR (400 MHZ; DMSO) 0.785-1.471 ppm (CH_3/CH_2), 2.12 ppm (CH_2), 3.071-4.591 ppm (CH_2), 4.966-5.032 ppm (NCH/OCH/C=C-H/CH), 6.098-6.178 ppm (hydroxyl proton of sugar), 6.770-7.138 ppm (aromatic protons), 7.8 ppm (NH2).

Conjugate AP1: Color: colorless, yield: 70%, m.p.: 124-128°C, Rf: 0.42, IR: aldehyde at 1739 cm−1, NH (amide) at 2923 cm^{-1}, NH (amine) at 3288 cm^{-1}, OH at 2853 cm^{-1}, CO (aromatic) 1222 cm^{-1}, DSC: Tg 130°C, NMR: 1H NMR (400 MHZ; DMSO) 0.8-1.247 ppm (CH_3), 2.30-4.895 ppm (CH_2), 4.860-5.045 ppm (NCH/OCH/C=C-H/CH), 5.859 ppm (hydroxyl proton of sugar), 6.880-7.164 ppm (aromatic protons), 9.535 ppm (NH2).

Conjugate AC1: Color: pale yellow, yield: 76%, m.p.: 88-92°C, Rf : 0.61, IR: aldehyde peak at 1629 cm^{-1}, NH (amide) at 2925 cm^{-1}, NH (amine) at 3274 cm^{-1}, OH at 3345 cm^{-1}, CO (aromatic) 1241 cm^{-1}, DSC: Tg 96°C, NMR: 1H NMR (400 MHZ; DMSO) 0.833-1.499 ppm (CH_3), 2.494-3.320 ppm (CH_2), 3.555 ppm-4.20 (hydroxyl proton of sugar/OCH/OCH_2/NCH), 6.826-7.172 ppm (aromatic protons), 8.178 ppm (NH_2).

- Toxicity assay

The cytotoxicity of manufactured Atenolol conjugation was investigated using a brine shrimp lethality bioassay [16,17]. By comparing the mean surviving larvae of the test and control tubes, the % lethality was calculated. The best-fit line plotting concentration versus percentage lethality yielded LC50 values. The three Atenolol conjugates showed no appreciable toxicity, with substantially higher LC50 values indicating an excellent safety profile. Table 2 summarises the toxicity results.

Table 2.Results of Toxicity Study of ATN-MS Conjugates.

Drugs	Conc. of compound µg/ml	Total no. shrimps used/tube	Shrimp survived			Total No. of Shrimp Survived	Percentage mortality	LC_{50} (µg)	(95% confidence interval)
			T1	T2	T3				
AG1	50		8	9	8	25	13.79	323.41 ± 10.00	305.23-339.58
	100		8	8	8	24	13.21		
	150		7	7	7	21	27.58		
AP1	50	10	8	8	7	23	20.68	340.81 ± 10.50	323.76-357.85
	100		7	8	8	23	16.91		
	150		7	6	7	20	31.03		
AC1	50	10	8	7	7	22	20.68	334.81 ± 10.50	318.06-351.55
	100		8	8	8	24	16.84		
	150		6	7	7	20	31.03		

- Cellular uptake study

H9c2 rat heart cells were subjected to RP-HPLC analysis to determine the quantity of Atenolol in Atenolol-saccharide conjugates [14,15]. The concentration of Atenolol present in the cells is determined by the strength of the peak observed at a certain retention time. Atenolol and its saccharide conjugates were introduced to the cells, incubated, rinsed, then carefully removed from the adherent cells with cold PBS. Furthermore, chromatographic procedures ensured that medications from chemically disturbed cells could be analysed.In view of results in Figure 1, Atenolol concentration in H9c2 rat heart cells was observed to contain 25 to 40 µg/ml after being loaded with 50 µg/ml of Atenolol and Atenolol saccharide conjugates. The reason behind the accumulation of the drug in cells was supposed to bind drug saccharide conjugates at receptors, as evidenced by docking analysis. Further, improvement in intracellular drug transport was also claimed due to bypassing the drug transport system so as to favor a dynamic balance between intra and extracellular concentration of drug during an extended period of incubation. Figure 2 shows the percentage of drug uptake in H9c2 cells after 12 h incubation was showed in the range of 40% to 53%. The chemical conjugation of Atenolol and various saccharide units influenced drug permeation across cells and ensured drug stability across cells. The individual HPLC chromatograms of Atenolol and Atenolol-saccharide conjugates are after treatment of H9c2 rat heart cells are mentioned in Figure 3.

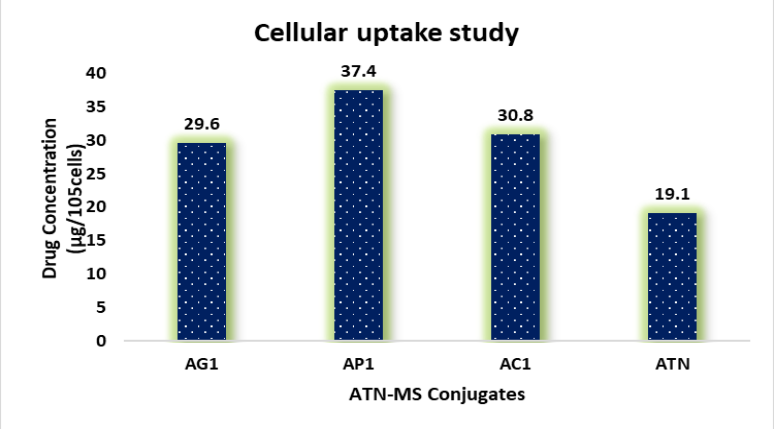

Figure 1. Cellular uptake of ATN-MS conjugates in H9c2 rat heart cells.

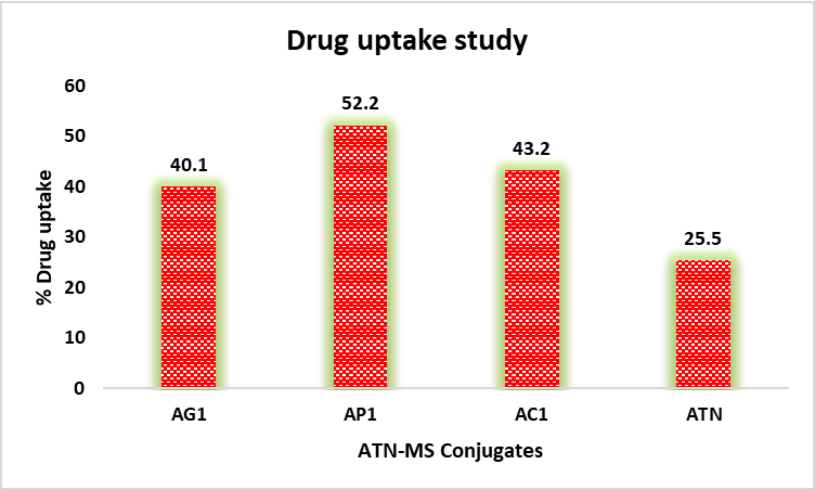

Figure 2. Drug uptake of ATN-MS conjugates in H9c2 rat heart cells.

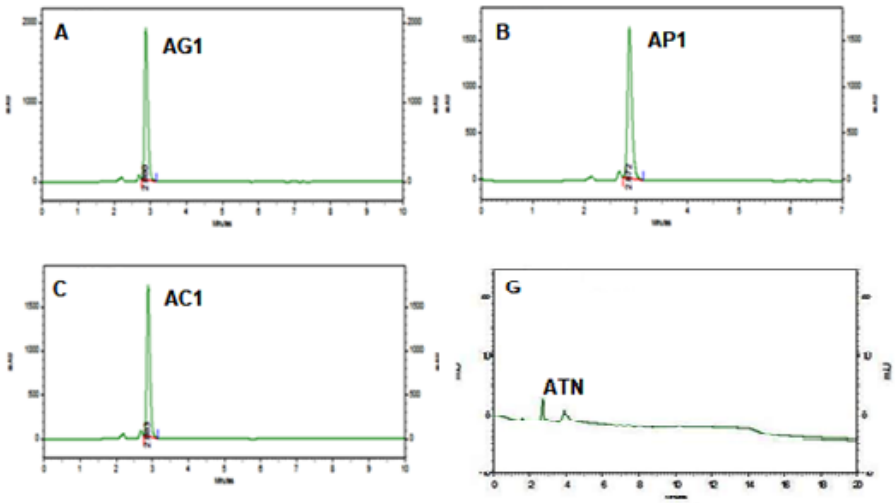

Figure 3. HPLC chromatogram of ATN-MS conjugates A) AG1, B) AP1, C) AC1 and D) and D) ATN in H9c2 rat heart cells.

Impact

Delivering therapeutically adequate doses of the drug directly to the site of action not only improves the efficacy, it also prevents high doses reaching other organs thereby significantly reducing cytotoxic effects of the drugs on other organs. Targeted delivery has the potential to revolutionize current treatments and improve the clinical outcome in cardiovascular patients. This approach can reduce the frequency of dosing, thereby improving the patient compliance and reducing the cost of therapy. This could be an important socio-economic contribution of the study for the society.

Conclusions

Various chemically modified saccharides have been effectively conjugated to Atenolol in order to improve drug availability to cardiac cells for better treatment of cardiovascular diseases and a reduction in adverse effects. The modified saccharides had a higher lipophilicity and, curiously, better swelling properties. This discovery opens

up the possibility of using modified saccharides as an excipient in extended-release medication formulations. FTIR and DSC were used to characterise the chemically changed saccharides with the cardiovascular medication. The conjugates that have been synthesised have been shown to be stable. The medication can be successfully targeted for selective cardiac administration, according to the results of cell line tests.The toxicity research demonstrates that the produced chemicals are safe to use. As a result, conjugating Atenolol with a biodegradable and biocompatible polymeric system to increase drug delivery selectivity for the treatment of cardiovascular disorders appears to be a potential way to improve drug delivery selectivity.

Conflicts of Interest

The authors declare no conflict of interest.

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