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Tolbutamide induces conformational change and promotes albumin glycation

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Abstract

Tolbutamide is a first generation sulfonylurea drug used for the treatment of type II diabetes. In the present study, the effect of Tolbutamide on albumin conformation was investigated using BSA as a model protein. The binding of Tolbutamide was found to induce a conformational change in the albumin, which was confirmed by Thioflavin T assay, ANS assay, and CD spectroscopy. Computer simulations suggested that the binding of Tolbutamide increases the solvent accessibility of lysine residues, the hotspots of glycation. Furthermore, the change in conformation of albumin facilitates increased glycation, which was observed by AGE specific fluorescence and the results were corroborated by mass spectrometric analysis. This study suggested that Tolbutamide enhances albumin glycation by inducing conformational change in the BSA and hence could be a risk factor if used for prolonged period.

Keywords

Bovine Serum Albumin, Diabetes, Glycation, Protein conformation, Tolbutamide.

Abbreviations

AGEs, Advanced Glycation End Products; ANS, 1-anilino-8-naphthalenesulphonate; BSA, Bovine Serum Albumin; SASA, Solvent Accessible Surface Area

Introduction

Diabetes is a metabolic disorder characterized by elevated levels of plasma glucose. Prolonged glucose in diabetes results in several complications such as retinopathy, nephropathy, neuropathy and cardiovascular diseases.[1] One of the major causes of these complications is protein glycation and formation of advanced glycation end products (AGEs).[2] Glycation is a non-enzymatic reaction between carbonyl group of glucose and free amino group of protein, which further undergoes structural rearrangement to form advanced glycation end products.[3] Glycation of plasma proteins such as albumin, globulins, and fibrinogen have adverse effects which may include altered drug binding in plasma, free radical generation and activation of platelets.[4] Albumin is one of the most abundant plasma proteins having concentration of 3.5-5.5 g/dl in humans. It is also a major carrier protein, able to bind and transport endogenous solutes and broad range of drugs.[5] Drug binds albumin selectively at two main sites namely, Sudlow site I and Sudlow site II.[6] Site I, also called as warfarin binding site, is located on sub-domain IIA of albumin. Bulky heterocyclic compounds, for instance coumarins, sulfonamides, and salicylate, bind to this site.[7-9] Sub-domain IIIA contains Sudlow site II, which binds to aromatic carboxylic acids and profens.[8] The binding of drug to the albumin affect its pharmacologic and pharmacokinetic properties such as bioavailability, metabolism, and excretion.[10] Binding of some drugs also induces conformational change in the albumin.[11] There are numerous reports suggesting the conformational change in the albumin upon interaction with the drugs such as Phenothiazine Drugs, hydroxycinnamic acids, Troxerutin, tenoxicam, imipramine hydrochloride etc.[11-12]

Sulfonylureas are commonly used drugs for the management of type II diabetes, either alone or in combination with other drugs. Among the sulfonylurea drugs, Tolbutamide was the first to be used for the treatment of diabetes.[13] Earlier reports have suggested that Tolbutamide has very high affinity for serum albumin and ~ 90% drug is found to be protein

bound in circulation.[14] It is possible that the binding of Tolbutamide changes the conformation of albumin, and influence its glycation. Albumin has been reported to be one of the heavily glycated plasma proteins due to its abundance, longer half-life (21 days) and more number of lysine and arginine residues, the hotspots of glycation.[16] Therefore, in this study, we have investigated the effect of Tolbutamide on albumin conformation using BSA as a model protein.[14] We have found that the binding of Tolbutamide, a first generation sulfonylurea, induces significant conformational change in the albumin. It was proved by Thioflavin T assay, ANS assay, and CD analysis. Molecular dynamic simulations suggested that the binding of Tolbutamide increases the solvent accessibility of lysine residues of albumin. Furthermore, the change in conformation of albumin facilitates increased glycation, which was observed by AGE specific fluorescence and the results were corroborated by mass spectrometric analysis. This study suggested that Tolbutamide enhances albumin glycation by inducing conformational change.

Results and discussion

Tolbutamide binds to BSA

Binding of Tolbutamide to BSA was studied by fluorescence quenching assay. Figure 1 A shows the fluorescence spectra of BSA in the absence and presence of Tolbutamide. The BSA spectra showed maximum fluorescence intensity at 340 nm. Fluorescence intensity of BSA was found to be decreased with increasing concentration of Tolbutamide. Further the number of binding sites of Tolbutamide on BSA was calculated by using following equation.[15]

$$\text{Log } [(F0-F)/F] = \log K + n \log [Q]$$

Where, F0 and F are the fluorescence intensities in the absence and presence of Tolbutamide, Q is the concentration of Tolbutamide, K and n are binding constant and binding number, respectively. The graph of log [(F0-F)/F] vs log [Q] was plotted in Figure 1 B, in which the intercept and slope indicated the binding constant and binding number for Tolbutamide to BSA, respectively. The binding constant was found to be 3.2. The binding number was observed to be 2.2 which indicated that two molecules of Tolbutamide interact with single molecule of BSA.

Binding of Tolbutamide induces conformational change in BSA

The change in the BSA conformation due to binding of Tolbutamide was studied by CD spectroscopy, ANS and Thioflavin-T assay. CD spectra of BSA illustrated minima at 208 nm and 222 nm, a characteristic of α-helix.[25] It was observed that with increasing concentration of Tolbutamide, minima at these wavelengths was decreasing (Figure 2 A). Secondary structure analysis by CDPro software demonstrated that α-helix content decreases and β-sheets increase with increasing concentration of Tolbutamide. The turn and unordered structures did not differ much in presence of Tolbutamide (Figure 2 B). Furthermore, 8-Anilinonaphthalene-1-sulfonic acid (ANS), a commonly used fluorescent probe to study conformational change in proteins upon ligand binding was used.[26] A blue shift in the fluorescence emission maxima and increase in the fluorescence intensity signifies the conformational change and increased hydrophobicity of BSA. ANS showed emission maxima at 525 nm while ANS bound to BSA at various concentrations of Tolbutamide (0.1 M, 0.2 M and 0.3 M) showed blue shift with emission maxima at 470 nm. Increase in hydrophobicity of BSA was observed with increasing concentration of Tolbutamide (Figure 2 C). In addition to this, Thioflavin-T assay was performed to confirm the conformational change. Thioflavin T is a commonly used benzothiazole dye to diagnose amyloid fibrils that displays increased fluorescence upon binding to amyloid fibrils.[27] As Thioflavin T displays

enhanced fluorescence upon binding to β-sheet rich structures, increased fluorescence in Tolbutamide incubated BSA, suggested that Tolbutamide induces conformational change in albumin by increasing β-sheets (Figure 2 D). Hence, Thioflavin T assay results support the CD analysis and ANS assay results indicating that Tolbutamide induces conformation change by increasing β-sheets.

Tolbutamide binding exposes surface lysine residues and increases BSA glycation

As Tolbutamide is an anti-diabetic drug prescribed for chronic treatment, its ability to cause conformational change in albumin can influence its glycation. MD simulations were carried out to study the exposure of lysine residues, hotspots of glycation, after Tolbutamide treatment. The total solvent accessible surface area (SASA) of the lysine residues increased from 19800 (±81.7) $Å^2$ to 20050 (±75.0) $Å^2$ upon Tolbutamide binding, calculated within the VMD program with a spherical probe of 1.8 Å radius (Figure 3 A). Increment of solvent exposure of lysine residues is an indication of conformational change in BSA upon ligand binding. The increased exposure of lysine residues of BSA in the presence of Tolbutamide increases the probability of glucose to react with it and hence increases the chance of glycation. The validation of this result was done by *in vitro* glycation of BSA in the presence of Tolbutamide. AGE specific fluorescence was increased in presence of Tolbutamide (Figure 3 B). Additionally, the increase in glycation was confirmed by LC-MS/MS analysis using high resolution accurate mass spectrometer (Q-Exactive). Mass spectrometric data was analyzed by Proteome Discoverer. The peptides with glycation modification and good fragmentation pattern were selected. As expected the numbers of glycated peptides were more in glycated BSA as compared to native BSA, and this number increased in the presence of Tolbutamide (Figure 3 C). The detailed information about the modified peptides is given in supplementary Figure S1 and supplementary Table S1. Furthermore, the extent of modification in Tolbutamide treated BSA was quantified by extracted ion chromatograms

(XIC). For example, a representative hexose modified peptide (m/z 659.63269) was selected. Its MS/MS fragmentation pattern and XIC is shown in figure 4 A and 4 B respectively. Area under curve for the modified peptide was increased in case of Tolbutamide treated BSA as compared to glycated BSA. The additional XICs for the modified peptides are given in supplementary Figure S2. These results prove that glycation of serum albumin may increase in presence of Tolbutamide.

Conclusion

In our study, we have investigated the effect of Tolbutamide on the most abundant plasma protein, albumin. Tolbutamide induces the conformation change in BSA. Binding of Tolbutamide increases the hydrophobicity and β-sheets. Moreover, Tolbutamide was also found to enhance the solvent accessibility of lysine residues; site of glycation. The exposure of sites for glycation promotes the glycation reaction as evidenced by AGE specific fluorescence and LC-MS/MS analysis. Therefore in conclusion, the altered BSA structure exposes the sites for glycation and hence promotes this reaction. As a result, prolonged use of Tolbutamide may have adverse effects.

Experimental

Chemicals

All the chemicals were procured from Sigma-Aldrich unless otherwise mentioned.

Fluorescence quenching

BSA-Tolbutamide interaction was studied by measuring tryptophan fluorescence. BSA was titrated with different concentrations Tolbutamide (1-50 μM) and tryptophan fluorescence

was measured by excitation at 280 nm and emission was scanned from 300 nm to 500 nm. Background fluorescence from buffer and Tolbutamide were subtracted and graphs were smoothened.

Reaction for study of Tolbutamide-BSA binding

200 μM Tolbutamide was incubated with 50 mg/ml BSA in 0.2 M phosphate buffer, pH 7.4 for 24 h. Only drug and buffer served as a negative control. These samples were further used for Thioflavin T, ANS and CD analysis.

Circular Dichroism Spectroscopy

The far UV CD spectra (in wavelength range of 190-250 nm) of BSA (20 μg/ml) with or without various concentrations of Tolbutamide (0.1, 0.2, 0.3 M) was recorded using Jasco-J815 spectropolarimeter at ambient temperature. Each CD spectrum was accumulated from three scans at 50nm/min with cell path length of 0.1 cm. Contribution due to buffer was corrected in all spectra and observed values were converted to mean residual ellipticity (MRE) in deg cm^2 $dmol^{-1}$ defined as

$$MRE= M\theta_\lambda/10dcr$$

Where, M is the molecular weight of the protein, θ_λ is CD in millidegree, d is the path length in cm, c is the protein concentration in mg/ml and r is the number of amino acid residues in the protein. Secondary structure content of the BSA with and without Tolbutamide was calculated using the CDPro software (http://lamar.colostate.edu/~sreeram/CDPro/main.html).

ANS binding studies

Fluorescence measurements were performed with 1-anilino-8-naphthalenesulphonate (ANS) to determine binding of Tolbutamide to BSA after 24 h at RT. ANS is hydrophobic dye

which binds to solvent exposed hydrophobic regions in a protein. The final ANS concentration used was 10 μM, excitation wavelength was 375 nm and emission was monitored between 400 to 550 nm. Spectra were recorded at 100nm/min with slit widths of 7 nm each for excitation and emission monochromators on Perkin Elmer LS 50B luminescence spectrometer.

Thioflavin T assay

100 μM BSA with or without Tolbutamide was incubated with 20 μM Thioflavin T. Immediately, fluorescence was measured with excitation at 440 nm and emission was scanned from 460 nm to 550 nm on Perkin Elmer LS 50B luminescence spectrometer. Actual fluorescence of protein was measured by subtracting background fluorescence of the drug.

Molecular Dynamics (MD) Simulation

Initial coordinates of BSA molecule were taken from the crystallographic structure reported in the PDB database (PDB ID 4F5S). Tolbutamide was docked with BSA using Autodock.[17] The resultant system was immersed in a box of equilibrated TIP3P water molecules, maintaining a minimum distance of 14 Å from the box edge to each atom. Twenty two sodium counter ions were added at random positions within the simulation box. For comparison the free protein was also simulated. NAMD2.9 package[18] and CHARMM22 all-atom force field with CMAP correction was used for the simulation study.[19-20] Force field parameters for Tolbutamide were generated using the SwissPARAM tool.[21] Simulations were carried using a timestep of 2 fs in the isothermal-isobaric (NPT) ensemble at a temperature of 310 K and a pressure of 1 atmosphere. Each system was sampled for a total duration of 50 ns. The SHAKE algorithm[22] was used to constrain bond lengths involving hydrogen atoms. Constant temperature was maintained with Langevin dynamics with a collision frequency of 1 ps^{-1}, and constant pressure was maintained using the Langevin piston Nose- Hoover method

.[23] Three-dimensional orthorhombic periodic boundary conditions were employed and full electrostatics calculated with the particle-mesh Ewald method.[24] A non-bonded cutoff distance of 12 Å was employed, which were smoothened at a distance of 10.5 Å.

In vitro BSA Glycation

For glycation reaction, 50 mg/ml BSA was incubated with 0.1 M glucose with or without various concentrations of Tolbutamide (0.1, 0.2 and 0.3 M) in 0.2 M phosphate buffer containing 5 mM sodium azide, pH 7.4 for 15 days. Glycation associated fluorescence of BSA was measured in all samples at excitation of 370 nm and emission was scanned from 400-550 nm.

In-solution Trypsin Digestion

100 µg protein sample was kept overnight for acetone TCA (Trichloroacetic acid) precipitation after incubation of 15 days. Samples were then washed with chilled acetone and used for digestion. Samples were reduced and alkylated by 10 mM dithiothreitol for 30 min and 55 mM iodoacetamide for 45 min respectively. 2µg trypsin was added and the solution was incubated at 37°C for 16 h. 2 µL of formic acid was added to stop the reaction and then samples were vortexed and centrifuged. The peptides in the supernatant were collected and stored at -80°C until further used.

LC-MS/MS analysis

Tryptic peptides were fractionated using an Accela UPLC system (Thermo Scientific) connected to high resolution mass spectrometer Q-Exactive (Thermo Scientific). Peptides were injected and separated by Hypersil Gold C18-reverse phase HPLC column (150 X 2.1 mm, 1.9 mm) (Thermo Scientific) over a 45 min gradient, using 6 gradient segments (held at 2 % solvent A over 2 min, 2–40% A over 35 min, 40–98% A over 2 min, held at 98% A over

2 min, 98-2% A over 2 min held at 2% A for 2 min) with a flow rate of 350 µl min^{-1}. Solvent A was ACN with 0.1% formic acid and Solvent B was aqueous with 0.1% formic acid. Peptides were ionized by HESI ionization with a capillary temperature of 320°C. Tandem Mass Spectra were acquired using Q Exactive Orbitrap high resolution Mass Spectrometer controlled by Xcalibur software (Thermo Scientific). The Orbitrap was set to analyze the survey scans at 17,500 resolutions in the mass range *m/z* 350 to 1800 and the top five multiply charged ions in each duty cycle were selected for MS/MS fragmentation using higher-energy collisional dissociation (HCD).

Data processing and analysis

The raw data files were processed using Proteome Discoverer software (Thermo Scientific) and searched against the UniProt BSA sequence (P02769) using the SEQUEST HT algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS fragment ion mass tolerance was set at 0.5 Da. Search criteria included Hexose-162.053, Carboxyethyl lysine-72.021, Carboxymethyl lysine-58.005, FL-H_2O-144.042 and FL-H_2O-126.032 on lysine and arginine residues as variable modifications and carbamidomethylation of cysteine (+57.021) as static modification. Searches were performed with full tryptic digestion and a maximum of 3 missed cleavages were allowed. Peptide data was filtered to satisfy false discovery rate (FDR) of 5%. Annotation threshold was set at 5% of base peak and match tolerance was set at 0.05 Da. The amount of glycation was monitored by extracted ion chromatogram (XIC) of modified peptides using Xcalibur (Thermo xcalibur 2.2 SP1.48).

Statistical analysis

Statistical analysis was performed by Student's t-test. Data is expressed as mean ± SD. A p-value <0.05 was considered as statistically significant.

Acknowledgements

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Figure legends

Figure 1 A. Tolbutamide-BSA interaction study by fluorescence spectroscopy **B.** Plot of log [F0-F/F] vs log [Q] to determine binding number and binding constant.

Figure 2 Investigation of change in conformation due to Tolbutamide binding A. CD spectra **B.** CD Pro analysis **C.** ANS assay and **D.** Thioflavin-T assay of BSA without and with various concentrations of Tolbutamide.

Figure 3 Glycation in the presence of Tolbutamide A. Solvent Accessible Surface Area (SASA) analysis of lysine residues of BSA before and after Tolbutamide treatment by MD simulations **B.** AGE specific fluorescence assay **C.** A bar graph representing number of glycation modifications on BSA in the absence and presence of Tolbutamide by LC-MS analysis.

Figure 4 Detection of hexose modified peptide in Tolbutamide treated BSA A. MS/MS fragmentation and **B.** Extracted ion chromatogram (XIC) of modified peptide (m/z 659.63269) in glycated and Tolbutamide treated BSA.

Figure 1

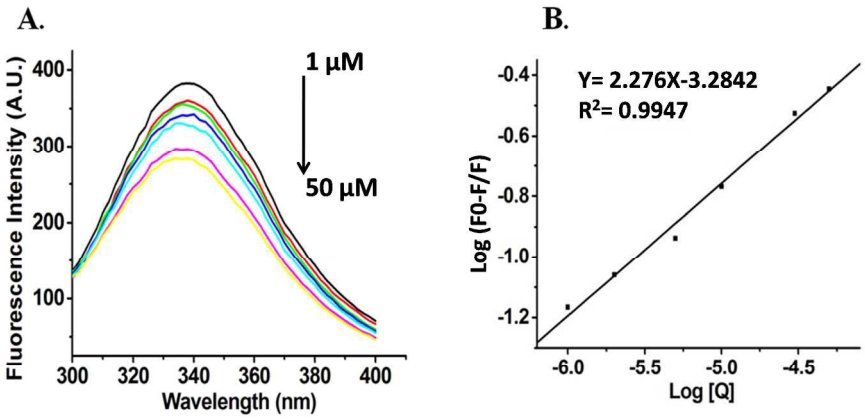

Figure 1 A. Tolbutamide-BSA interaction study by fluorescence spectroscopy B. Plot of log [F0-F/F] vs log [Q] to determine binding number and binding constant.
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Figure 2

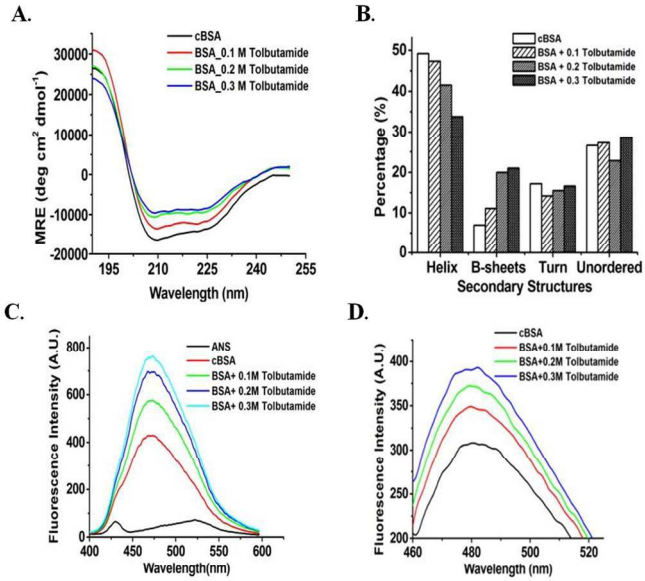

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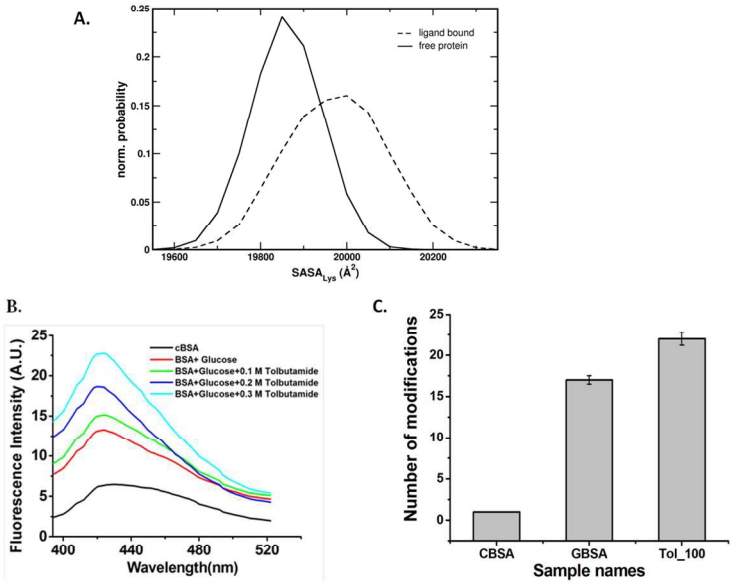
Figure 3



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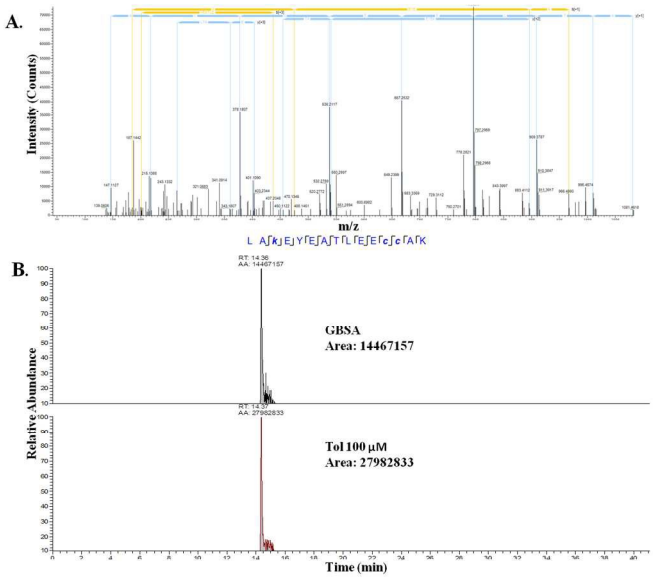

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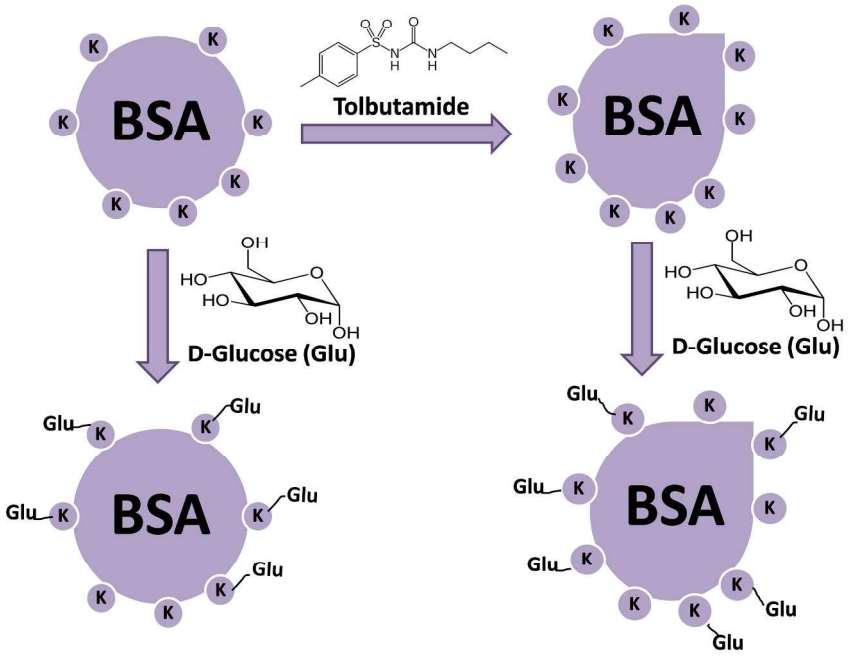
Graphical Abstract



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