Chapter

Putative Involvement of Thiol Protease Inhibitor in the Function of Alzheimer Drug

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Abstract

The intermolecular structure gets altered when drug-protein interaction takes place. It brings about alterations in the conformation of protein. An acetyl cholinesterase inhibitor (AChE) is the most used drug for patients who are suffering from Alzheimer's disease to curb its instigated symptoms. So, it is used as first-line defense in the insightful symptoms. This study is of concern with the interaction of cystatin purified from buffalo brain with its simple tri-step procedure including alkaline action, ammonium sulfate fractionation, and gel filtration chromatography on Sephadex G-75 with % yield of 64.13 and fold purification of 384.7. The inhibitor (brain cystatin (BC)) showed a single papain inhibitory peak drifted as single band on native PAGE; this purified inhibitor was interacted with donepezil to analyze the side effect of this drug since cystatin is an important regulatory protein that maintains the protease antiprotease balance. The conformational change was predicted when the UV spectra of cystatin was analyzed in the presence of donepezil contextual with the fluorescence spectra, but the fluorescence spectra showed 40 nm of red shift suggesting the change on interaction leading to a conclusion that donepezil is pertinent to imbalance to protease and antiprotease inhibitor perhaps the side effect of drug.

Keywords: acetyl cholinesterase (ach), acetyl cholinesterase inhibitor (AChE), Alzheimer's disease (AD), brain cystatin, (BC), thiol protease inhibitor

1. Introduction

Brain is exposed to a variety of neuromodulating agents given as therapy. The consequential action of these agents should be investigated to have the knowledge of their side influence. The primary degenerative disease of brain is dementia subsequently causing Alzheimer's disease. It is instigated in late life with cumulative properties like diminishing of memory, cognition, linguistic ability, and judgment. It is an advanced brain disorder relating to a person's inability to learn, reason, and carry out daily activities [1, 2]. The significant neurotransmitter acetylcholine is associated with normal functioning of the brain, and if the level of acetylcholine goes down in the cerebral cortex, this promulgates to Alzheimer's disease, the debilitating brain condition [3, 4].

Alzheimer's disease (AD) patients have lesser level of acetylcholine with the progressive abnormalities in cholinergic neurons. One line of attack to

reduce the impact of these abnormalities is to obstruct the relevant enzyme AChE (acetylcholinesterase) which acts as a foremost agent in the breakdown of acetylcholine (ACh) [2]. Acetylcholine hydrolysis into choline and acetate is prevented by AChE inhibitors in the synaptic clefts and ensuing in activating cholinergic transmission [3]. Donepezil (**Figure 1**) is a piperidine-class *(piperidine is a widely used building block and chemical reagent in the synthesis of organic compounds, including pharmaceuticals; piperidine is a widely used secondary amine)* AChE inhibitor, sensibly designed especially for Alzheimer's disease [5, 6]. It is used to improve cognitive function in patients of AD and shows no sign of hepatotoxicity [7–9]. The trade name of this drug is Aricept and it functions as an acetylcholinesterase inhibitor [10]. It has 100% of an oral bioavailability and easily passes the blood–brain barrier. Shows the half life of 70 hrs. The primary action of donepezil is by plummeting the breakdown of acetylcholine thus amassing the concentration of acetylcholine in the brain retaining back to its normal function [11].

The major neuropathological hallmarks of AD are senile plaque, neurofibrillary tangles, and neuronal loss. Both cathepsins and cystatins (cystatin A and B) are found in close association with senile plague, cerebrovascular amyloid deposits, and neurofibrillary tangles in Alzheimer's disease, Parkinson's, and patients suffering from senile dementia supporting the fact that they are amyloid constituents. Recent researches have shown that cathepsins are one of the most important proteases involved in the processing of neuropeptides in the central nervous system (CNS) of brain (**Figure 2**) [12], while cystatin C is also existing in high concentration and their concentration relevant in brain diseases. Cysteine proteinases in normal persons help in β-peptide clearing. The disturbance between the accumulative action of cathepsins and cystatins may lead to aggregation of potentially amyloidogenic fragment collection aggravates to form amyloid fibrils in nerve cells of AD patients causing cystatin concentration to shrink (**Figure 3**). Generated by imbalance of cathepsins and cystatin, these potentially amyloidogenic fragments at liberty into the extracellular space cause the aggravation of disease [12].

The powerful regulatory system is constituted by cystatins for endogenous cysteine proteinases (cathepsins) which are often permeable from the lysosomes of dying or diseased cells [13]. Cystatins are the natural inhibitors of cysteine proteases with wide occurrence in tissues and cells which belong to a super family of proteins [14]. Cystatin super family has been divided into three families on the basis of homology, inhibition of target enzymes, and presence or absence of disulfide bonds.

Family 1, also called as stefins, includes members of low-molecular-weight proteins (11 kDa) which lack disulfide bonds and carbohydrate contents. This family includes cystatins A and B and stefins C and D.

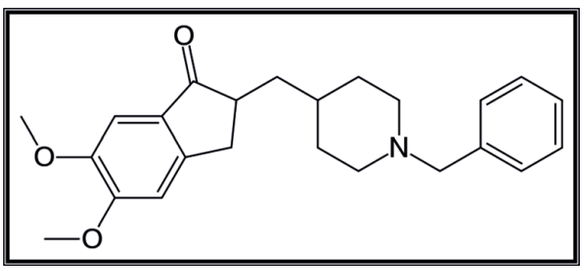

Figure 1.
Structure of donepezil [17].

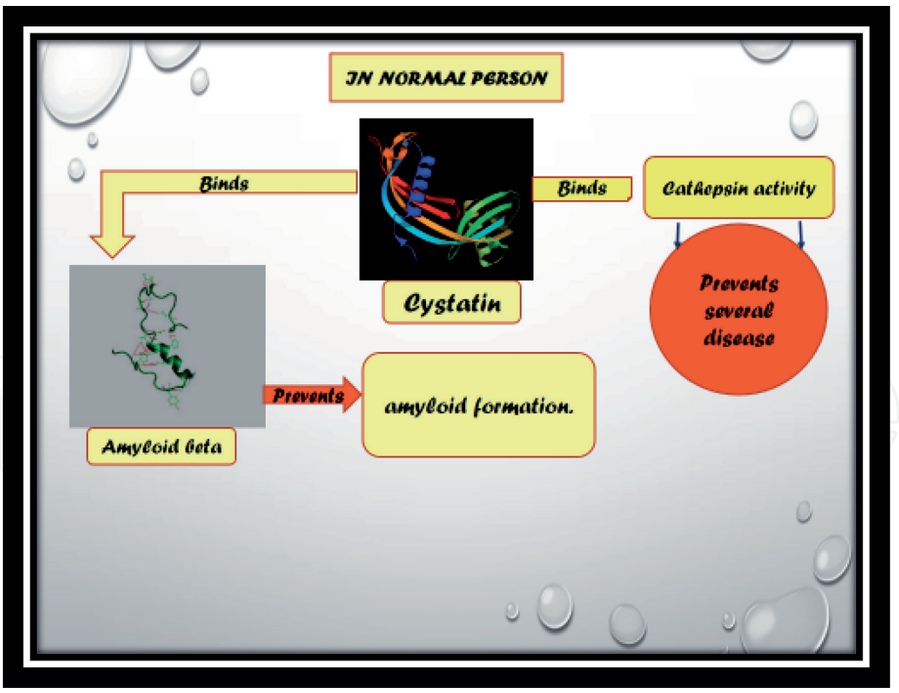

Figure 2.
Graphical abstract – Showing the function of cystatin in brain, how it is combating the several diseases.

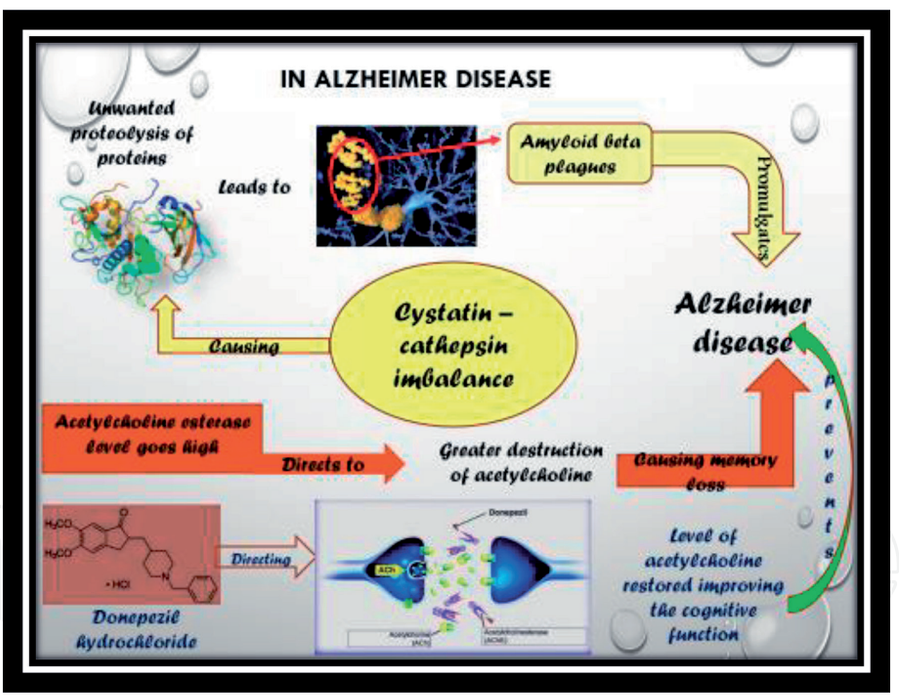

Figure 3.
Graphical abstract-2.

The figure shows the detailed interaction of cystatin-cathepsin imbalance and the role of acetylcholine esterase inhibitor as function in restoration of Alzheimer's diseases. They are found both in the body fluids and cells. Common illustration is cystatin C.

Kininogens or family 3 cystatins are large precursor molecules of the vasoactive kinins. They are glycoproteins of single chain that perform the multiple biological functions such as kinin delivery, induction of endogenous blood coagulation cascade, and acute phase response intercession. They are inhabitants of blood plasma [15].

Cystatins tightly bind and impede the activity of cathepsins; if the activity of cathepsins is not regulated, it instigates chronic diseases [13]. Senile plaque, cerebrovascular amyloid deposits, and neurofibrillary tangles in Alzheimer's disease are all the ramifications of imbalance between proteinases and their endogenous inhibitor cystatins [1].

Normal functioning of the brain is balanced by maintaining the level of acetylcholine and acetylcholinesterase inhibitor [16]. A previous report showed that donepezil binds along with HSA modifying it conformationally by effecting its free concentration in plasma [17].

The supplementation of donepezil was explored to find out any effect on cystatin (major regulator of thiol proteases: cathepsins B, H, and L, etc.) in the mammalian system. If the activity of these proteases is not controlled, it will lead to protease and antiprotease imbalance and repercussion to several diseases [18]. Therefore, it was thought worthwhile to investigate the donepezil cystatin binding and its role in the proper accomplishment of drug delivery and if it lead to kind of side effects as well as to gain knowledge about any conformational change in cystatin effecting its activity?

The study shows that the imbalance of protease-antiprotease purportedly leads the way to Alzheimer's disease, while the presence of drug donepezil unfolds cystatin which becomes unfit to bind cathepsins leading to a number of diseases as a considerable side effect of the drug. As cystatins play significant role in several diseases like, cancer and cardiovascular diseases [19]. Therefore, the usage of donepezil in such patients requires additional attention.

2. Materials

Papain (99% purity) was obtained from Sigma Chemical Company (St. Louis, USA). Donepezil (an Alzheimer drug) was purchased from Ranbaxy (India). The solutions were prepared in 50 mM phosphate buffer of pH 7.4. Salts were purchased from Merck (India). The protein concentration was determined spectrophotometrically. All other reagents were of analytical grade, and double distilled water was used throughout.

3. Methods

Purification of brain cystatins. Buffalo brain whole mass (150 g) was brought fresh from slaughter house in a box containing ice packs. It was carefully washed and rinsed with water, eliminating thin membrane and nerves by forceps, and the whole brain tissue was homogenized in 50 mM sodium phosphate buffer (300 mL) of pH 7.5 containing 0.15 M NaCl, 3 mM EDTA, and 2% n-butanol. After centrifugation at 11,000 rpm for 15 min at 40°C, residue was cast off, and the supernatant was further processed. The procedure involved a combination of alkaline treatment (pH 11.0), ammonium sulfate fractionation, and gel filtration chromatography. The brain was homogenized and fractionated with ammonium sulfate between 40 and 60% saturation; the precipitated protein was then dialyzed against 50 mM sodium phosphate buffer pH 7.4 containing 0.1 M NaCl. Elution profile showed two protein peaks—one major and one minor called as peak-I and peak-II. Peak-I is conforming to high-molecular-weight buffalo brain cystatin with significant inhibitory activity and protein content; however, peak-II with insignificant protein concentration and low inhibitory activity was not taken into consideration for further studies. Peak-I named as BC was purified with fold purification of 384.72 and yield of 64.13%.

Papain inhibitory fractions of peak-I were pooled, concentrated, and checked for purity. Five milliliter fractions were collected and assayed for protein and cystatin activity. Homogeneity of the preparation was investigated by 7.5% PAGE [20].

4. Spectroscopic studies

4.1 Fluorescence spectra of brain cystatin with drug

Brain cystatin (BC) (1 μM) was incubated for 30 min with subsequent higher concentration of donepezil in 0.05 M sodium phosphate buffer pH 7.5 in a final reaction volume of 1 mL at room temperature. The same buffer is used for preparation of drug solutions. Fluorescence measurements were carried out on a Shimadzu Spectroflourimeter model RF-5301PC (Shimadzu, Japan) equipped with a 150 W Xenon lamp and a slit width of 10 nm at 298 K. The fluorescence was recorded in wavelength region 300–400 nm after exciting the protein at 280 nm. The slits were set at 10 nm for excitation and emission. The path length of the sample was 1 cm.

4.2 UV spectra of cystatin in the presence of donepezil

The UV measurement of brain cystatin in the presence and absence of drug was made in the range of 200–300 nm, and the inhibitor (cystatin) concentration was fixed at 1 μM, while the drug concentration was varied from 0.16 to 1.6 μM. Absorption spectra were recorded on a double-beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.

4.3 Activity measurement of brain cystatin in the presence of donepezil

The inhibitory activity of the purified inhibitor (BC) under native conditions was assessed by its ability to inhibit caseinolytic activity of papain by the method of Kunitz [21]. The inhibitor (1 μM) was incubated with increasing concentrations of donepezil at 25°C for 30 min before the activity was measured. Activity of untreated BC was taken as 100%.

5. Results

5.1 Interaction of donepezil with brain cystatin

Alzheimer's disease is a progressive brain disorder that gradually destroys a person's memory and ability to learn, reason, and make judgments. AChE is responsible for degradation of the neurotransmitter acetylcholine (ACh) in the synaptic cleft of neuromuscular junctions and of neuronal contacts in the central nervous system [22, 23]. Donepezil belongs to the important class of acetylcholinesterase inhibitors (AChEIs) [24]. The results of the interaction of donepezil with cystatin are given below.

5.2 Intrinsic fluorescence studies of cystatin in the presence of donepezil

Cystatin (1 μM) was incubated with various concentrations of donepezil varying from 2 to 10 μM for 30 min. The fluorescence was recorded in the wavelength region of 300–400 nm after exciting the protein solution at 280 nm for total protein fluorescence. Donepezil caused unfolding of the cystatin as indicated by

enhancement in fluorescence intensity accompanied by the red shift of 40 nm as compared to –max of native cystatin (340 nm), while the drug (native) shows –max at 370 nm. However at 1.6 μM, when it forms complex with cystatin, there was a shift in –max of 10 nm with significant enhancement in fluorescence intensity (**Figure 4**).

Cystatin (1 μM) was incubated with various concentrations of donepezil varying from 0.16 to 1.6 μM for 30 min. The fluorescence was taken in the range of wavelength 300–400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 10 nm for excitation and emission. The path length of the sample was 1 cm in the final reaction volume of 1 mL in 0.05 M sodium phosphate buffer pH 7.5.

5.3 UV-vis spectra of cystatin in the presence and absence of donepezil

Cystatin concentrations were fixed at 1 μM, while the donepezil concentrations varied from 0.16 to 1.6 μM. Absorption spectra of native cystatin and in the presence and absence of donepezil were recorded in the range of 200–300 nm. The UV absorption intensity of cystatin increased with increasing concentration of donepezil concentration; however, the slight decrease in absorption intensity may be due to disruption or perturbation of absorbing groups (**Figure 5**).

5.4 Inhibitory activity of cystatin in the presence of donepezil

A change in the inhibitory activity of cystatin with increasing concentration of donepezil is shown in **Table 1**. The effect of donepezil on cystatin function was assessed by monitoring its changes in antiproteolytic activity by caseinolytic assay of papain [21]; 1 μM of cystatin was incubated with increasing concentration of

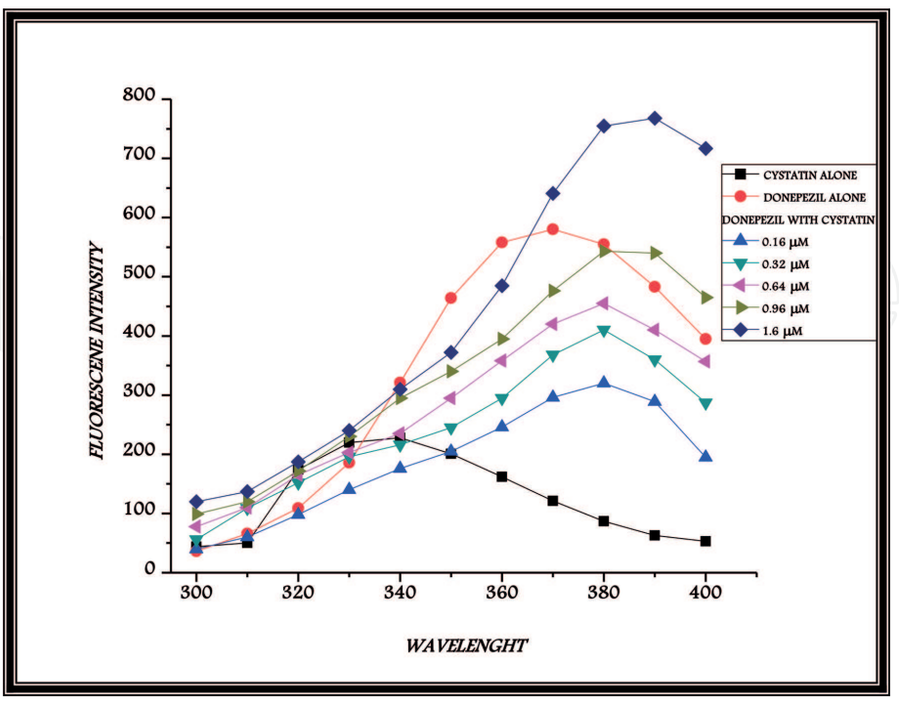

Figure 4.
Fluorescence spectra of cystatin in the presence and absence of donepezil. Cystatin (1 µM) was incubated with various concentration of Donepezil varying from 0.16 to 1.6 µM for 30 min. The fluorescence was recorded in the wavelength region 300–400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 10 nm for excitation and emission. The path length of the sample was 1 cm in the final reaction volume of 1 ml in 0.05 M sodium phosphate buffer pH 7.5.

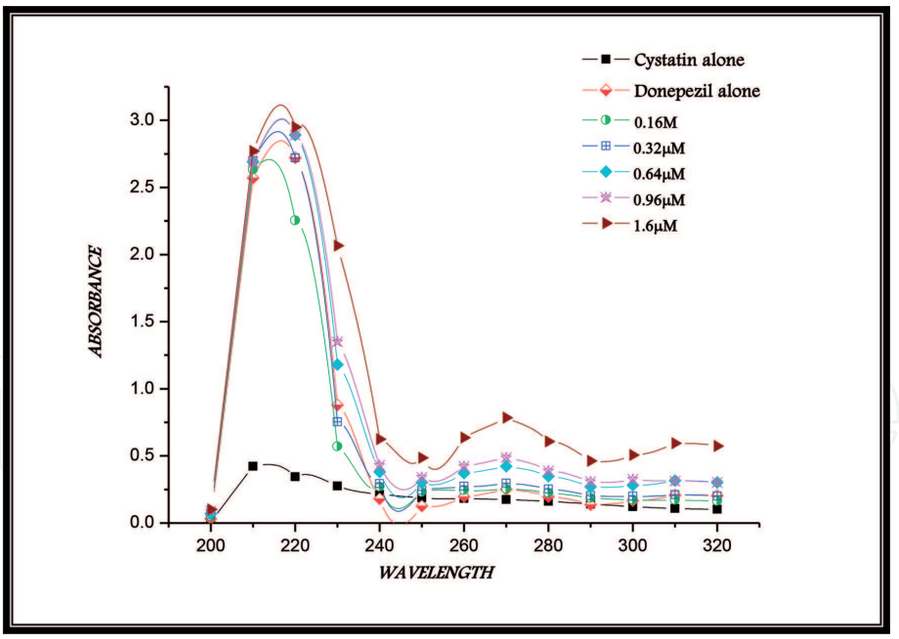

Figure 5.
UV-vis spectra of cystatin in the presence and absence of donepezil. Cystatin concentrations were fixed at 1 μM, while the donepezil concentration was varied from 0.16 to 1.6 μM. Absorption spectra of native cystatin in the presence and absence of donepezil were recorded in the range of 200–300 nm cuvette of 1 cm path length for 30 min in the final reaction volume of 1 ml in 0.05 M sodium phosphate buffer pH 7.5.

donepezil (0.16–1.6 μM). Exposure of cystatin to increasing concentration of donepezil resulted in rapid decline of antiproteolytic activity; 85% decline in the activity was seen at 1.6 μM of donepezil with more than half of the inactivation of cystatin which was taking place at concentration as low as 0.32 μM.

Table 1 shows changes in the inhibitory activity of brain cystatin after its incubation for its inhibitory activity with increasing concentrations of donepezil. Cystatin (1 μM) was treated with varying concentrations of donepezil (0.16–1.6 μM) for 30 min in the final reaction volume of 1 mL in 0.05 M sodium phosphate buffer pH 7.5.

5.5 2-D gel electrophoresis

Purity of brain cystatin was confirmed by 2-D gel electrophoresis; purified cystatin was run in one dimension on the isoelectric focusing, and after this, it was run in second dimension on 12.5% PAGE. On 2-D gel electrophoresis, BC migrated as a single

S.no	Drug concentration	% Remaining inhibitory activity of cystatin
1	Cystatin alone	100
2	Cystatin + 0.16 μM donepezil	57 ± 0.623
3	Cystatin + 0.32 μM donepezil	40 ± 0.938
4	Cystatin + 0.64 μM donepezil	38 ± 0.772
5	Cystatin + 0.96 μM donepezil	24 ± 0.932
6	Cystatin + 1.6 μM donepezil	15 ± 0.680

All data are expressed as mean ± SE for three different sets of experiments; statistical significance was conducted employing one-way ANOVA. A probability level of 0.05 was selected showing that results are significant.
The table shows changes in the inhibitory activity of brain cystatin after its incubation for its inhibitory activity with increasing concentrations of donepezil. Cystatin (1 μM) is treated with varying concentrations of donepezil (0.16–1.6 μM) for 30 min in the final reaction volume of 1 ml in 0.05 M sodium phosphate buffer pH 7.5.

Table 1.
Inhibitory activity of cystatin in the presence of donepezil.

Figure 6.
2-D gel electrophoresis of purified BC: after isoelectric focusing, IPG was run horizontally over 12.5% gel between the glass slabs. A single dot was obtained toward the negative side of the IPG strip.

dot further supporting the purity nature of BC. The position of the spot was approximately in the range of 7–8 pH on IPF strip (pH 3–10) suggesting the pl of BC as 7–8.

All data are expressed as mean ± SE for three different sets of experiments; statistical significance was conducted employing one-way ANOVA. A probability level of 0.05 was selected showing that results are significant (**Figure 6**).

6. Discussion

The drug inducing changes in protein function leading to adverse side effects is the area of continual scientific investigation [25, 26].

Even small structural differences in protein conformation can lead to drastic changes in functional parameters [26]. Addition of small molecules such as many drugs, particularly those with local anesthetics, tranquilizers, and antidepressants, can bind to the native state and can alter the delicate balance of various interactions in proteins [27–30].

The adverse drug reactions are triggered due to gathering of drug molecules at localized sites in the body causing their elevated concentration [31] and ligand-induced protein structure conformational changes [32]. The drugs used as medical therapy are unable to act in the specified area because of these intricate mechanisms; therefore, all the varied external parameters are taken into account which combine the study of the conformational modifications in proteins along with drugs resulting complexes causing any side effect which are paramount for the study. These studies enable us to understand how ligand affinity can be planned and how the protein conformation upon complexation can be managed [26] which is decisive in a massive range of imperative biochemical phenomena.

In the present work and structural and functional analyses of cystatin, a protein ubiquitously present in mammalian cells and tissues was studied which showed a significant increase in fluorescence intensity due to unfolding of cystatin in the presence of donepezil (**Figure 4**). Such kind of changes have also been documented earlier, after interaction of ligands (phytohormones, cytokinins, abscisic and gibberellic acids) with wheat germ agglutinin resulting in 60% increase in fluorescence intensity of native protein [33].

Donepezil-cystatin complexation showed 40 nm red shift in λ_{max} indicating exposure of aromatic residues to the solvent caused by conformational changes in the protein [34, 35]. Absorption spectral measurements of cystatin in the presence of drug showed a peak noticeable at 275 and 210 nm in spectra obtained at 0.16 μM donepezil concentration (**Figure 5**). Suggesting changes mainly due to tryptophan and tyrosine residues [36].

Thus, the results indicate that the UV absorption and fluorescence emission changes in donepezil-mediated interaction are due to conformational changes in cystatin mainly arising from interaction affecting the chromophoric groups of the protein which produce significant effect on the activity of cystatin. The study shows that in the presence of donepezil, cystatin gets unfolded which is a side effect of donepezil.

There is a gradual decline in the cystatin activity with increasing drug concentration resulting in 62% loss at 0.64 μM donepezil (**Table 1**). Further magnitude of decline was relatively smaller with increasing drug concentration up to 1.6 μM. Cystatin retained only 15% of its antiproteolytic potential.

The knowledge about the pharmacokinetics and pharmacodynamics of the drug-protein interaction continues to expand. The increased information available to clinicians might help in optimizing the use of these agents in the management of patients with Alzheimer's disease and other diseases. The clinical utility of measuring these parameters in daily practice awaits further research [37].

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