Proteolytic Imbalance in Depression as Potential Pathological Trait of Diseases

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Abstract: Cystatins are the thiol Proteinase inhibitors, present ubiquitously in mammalian body. They prevent unwanted proteolysis and play important role in several diseases. Regulation of cysteine Proteinase and their inhibitors is of utmost importance in diseases like Alzheimer, amyloid angiopathy and other neurodegenerative disease. Haloperidol is an antipsychotic drug used in the treatment of schizophrenia. In this paper interaction of brain cystatin (BC) with haloperidol has been studied by UV absorption and fluorescence spectroscopy to explore the drug induced changes in functional and structural integrity of the brain cystatin. The fluorescence quenching data analysed by stern vover analyses indicated the presence of static component in the quenching mechanism. The thermodynamic parameters ΔG° (Free energy change) found to be -39.37 KJ/mol revealed that both hydrogen bonds and hydrophobic interactions played a major role in the binding of Haloperidol with BC. This binding leads to decrease in the activity of cystatin showing its involvement in protease-antiprotease imbalance. It may also be taken as the side effect of the drug haloperidol, such studies may be used as a tool for drug designing.

Keywords: Haloperidol, Cystatin, Antidepressant, Neurotransmitter, Purification of Buffalo Brain Cystatin, Thiol Proteinase Inhibitors, depression schizophrenia.

Abbreviations:
BC: Brain Cystatin, MAOI (monoamine Oxidase inhibitor; antidepressant), MAO (monoamine amine Oxidase)

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Research field: Proteinase inhibitor

Acknowledgments: Facilities provided by Aligarh Muslim University are gratefully acknowledged.

1. Introduction

An antidepressant is a psychiatric medication used to alleviate mood disorders, such as major depression. Drugs including the monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), and serotonin-nor epinephrine reuptake inhibitors (SNRIs) are most commonly used antidepressant associated with the term. Despite the name, antidepressants are often used to treat other conditions, such as anxiety disorders, obsessive compulsive disorder, eating disorders and chronic pain. Although the mechanisms of the action of these antidepressants are not precisely understood, their principal target of action is at the monoamine transporter proteins located at nerve endings. Monoamine neurotransmitter transporters act to terminate synaptic neurotransmission. Drugs such as SNRIs produce their effects on the central nervous system via their actions on synaptic transmission. In general, these drugs work on a very limited number of transmitters, including catecholamines (dopamine and norepinephrine), serotonin (5-hydroxytryptamine [5-HT]), and acetylcholine. [18].

Depression affects 12 to 17% of the population during the lifetime of most Americans. In vitro studies evaluating the properties of SNRIs show that how the drug binds to the receptor (via receptor binding assays). In an effort to determine the mechanism of action of the antidepressants, and binding studies are used to determine the ability of a drug to attach to a particular protein [18].

Haloperidol is a typical antipsychotic drug [22] it comes under the class of SNRI [Fig-1] it is used in the treatment of schizophrenia as well as, in the treatment of acute psychotic states and delirium. Due to its strong central antidopaminergic action, it is classified as a highly potent neuroleptic drug and possesses a strong activity against delusions and hallucinations, the peripheral antidopaminergic effects of haloperidol accounts for its strong antiepinephrine activity. [12].

Cysteine proteases comprise a group of proteolytic enzymes that cleave the peptide bonds by the use of a reactive cysteine residue at the catalytic site. The action of these proteolytic enzymes is biologically controlled by proteinase inhibitors. Namely cystatins they constitute a powerful regulatory system for endogenous cysteine proteinases which are often secreted or leaking from the lysosomes of dying or diseased cells. Cystatins are proteins that tightly bind and inhibit cysteine proteinases to elevate their the harmful effect [21]. It is well known that cysteine proteases are implicated in various pathologies of the brain. Alzheimer’s disease and many other neurodegenerative disorders are associated with the accumulation of abnormal protein assemblies in the central nervous system. [2] Cystatin C also found to be linked with epileptogenesis and epilepsy [19] along with other neurodegenerative diseases [23].

Fluorescence spectroscopy is a powerful tool for the study of the reactivity of chemical and biological systems since it allows nonintrusive measurements of substances in low concentration under physiological conditions [11]. Because
of its high sensitivity, rapidity, and simplicity [3], fluorescence technique has been widely used for drug–protein studies [17,4,5]. In this paper, the interaction between BC and Haloperidol was studied by spectroscopy including fluorescence, UV–vis absorption and antiproteolytic assay

The drug is rapidly absorbed. Plasma-levels reach their maximum within 20 minutes after injection. The bioavailability is 100% and the very rapid onset of action is seen within about ten minutes. The duration of action is 3 to 6 hours. Plasma levels of 4 micrograms per liter to 20 micrograms per liter are required for therapeutic action. Haloperidol is also used in the control of the symptoms of acute psychosis, hyperactivity and aggression. [8]. since it binds with cystatin the effectiveness of the drug may be reduced as well as it may leads to side effect to of drug thus producing protease antiprotease imbalance

Figure 2: Graphical presentation of proposed work

2. Material and Methods

2.1 Materials

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

2.2 Purification of Brain Cystatin

Fresh brain tissue (150 grams) was homogenized in 50 mM sodium phosphate buffer of pH 7.5 (30 ml) containing 1% NaCl, 3mM EDTA and 2% n-butanol. After centrifugation at 11000rpm for 15 minutes at 4°C residue was discarded and the supernatant was further processed. The procedure involved a combination of alkaline treatment (pH 11.0), ammonium sulphate fractionation and gel filtration chromatography. Buffalo brain was homogenized and fractionated with ammonium sulfate between 40-60%, it 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 named as peak-I and peak-H. Peak-I corresponding to high molecular weight. Cystatin had significant inhibitory activity and protein content; however peak-II with insignificant proteins concentration and low inhibitory activity was not taken into consideration for further studies. Peak-I renamed as BC was then 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. Homogeneity of the preparation was investigated by 7.5% PAGE. [1]

2.3 Spectroscopic Studies

Fluorescence spectra of brain cystatin with haloperidol.

Brain cystatin (BC) (1µM) was incubated for 30 min with increasing concentration of Drug in 0.05 M sodium phosphate buffer of pH 7.5.
phosphate buffer pH 7.5 in a final reaction volume of 1ml at room temperature. Drug solutions were prepared in the same buffer. Fluorescence measurements were carried out on a Shimadzu Spectrofluorimeter model RF-5301PC (Shimadzu, Japan) equipped with a 150 W Xenon lamp and a slit width of 10 nm at 298K. 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. The data was analyzed by stern-Volmer equation

\[
\frac{F_0}{F} = 1 + K_{sv} [Q]
\]

Where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of quencher respectively, \( K_{sv} \) the stern-volmer quenching constant and \([Q]\) is the concentration of the quencher.

Determination of binding constant \( K \) and number of binding sites \( n \)

When small molecules binds independently to set of equivalent sites on a macromolecules, the equilibrium between free and bound molecules is given by the following equation \[20,13\]

\[
\log \left( \frac{F_0 - F}{F} \right) = \log K + n \log [Q]
\]

Where \( K \) and \( n \) are the binding constant and number of binding sites respectively thus a plot of \( \log \left( \frac{F_0 - F}{F} \right) \) versus \([Q]\) can be used to determine \( K \) as well as \( n \).

UV spectra of cystatin in the presence of antidepressant

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

Activity measurement of brain cystatin in the presence of drug haloperidol

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 [Kunitz (1947)]. The inhibitor (1 µM) was incubated with increasing concentrations of drugs at 25°C for 30 min before the activity was measured. Activity of untreated BC was taken as 100%.

3. Result

Fluorescence spectra of Haloperidol with brain cystatin 1µM of BC treated with increasing concentration of Haloperidol (0.02-0.1µM) and was subjected to fluorescence spectroscopy to assess the effect on conformation. The protein samples were excited at 280nm and emission range was 300-400nm. Haloperidol at 0.02µM did not induce any change in emission \( \lambda_{max} \) however 68% decline in fluorescence intensity was observed. Beyond this concentration (0.08µM) profound decline in fluorescence intensity (75%) was observed. Significant quenching in parent protein was observed (95%) when cystatin was incubated with 0.1µM of Haloperidol. [Fig-3].

Percent decrease in tryptophan fluorescence in the presence of Haloperidol with cystatin

<table>
<thead>
<tr>
<th>BC alone fluorescence</th>
<th>Drug alone 0.02µM</th>
<th>Drug alone 0.04µM</th>
<th>Drug alone 0.06µM</th>
<th>Drug alone 0.08µM</th>
<th>Drug alone 0.1µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>98</td>
<td>68</td>
<td>77</td>
<td>69</td>
<td>75</td>
</tr>
</tbody>
</table>

\[
\Delta G^0 = \frac{-RT}{n} \ln \left( \frac{K_{sv}}{K_{sv} + [Q]} \right)
\]

For fluorescence quenching, the decrease in intensity is usually described by the well-known ster-Volmer equation. The value of \( K_{sv} \) was found to be 15 x10^6 mol \(^{-1}\) for Haloperidol [Table-1].

Determination of binding constant \( K \) and number of binding sites \( n \)

The value was determined as described earlier in amitriptyline section. The binding constant and the number of binding sites was found to be 7.988 x10^6 mol \(^{-1}\) and 1.1 respectively [Table-1].

\[
\Delta G^0 \text{ of interaction between Haloperidol with Cystatin}
\]

The forces of interaction between drugs and biomolecules include hydrophobic force, electrostatic interactions, vander Waals interactions and hydrogen bonds. In order to identify the interacting forces between amitriptyline with cystatin, the thermodynamic parameters i.e. free change (\( \Delta G^0 \)) of the
interactions were calculated from the following equations:

\[ \Delta G^o = -RT \ln K \text{ (KJ/mol)} \]

Free energy change (\( \Delta G^o \)) of the interactions was calculated from the equations was -39.37 KJ/mol. \([\text{Table-1}]\) indicating the reaction to be spontaneous.

**Figure 4:** Stern-Volmer plots for the binding of BC to haloperidol at different concentrations at 298 K.

**Table 2:** Different Parameter of the Drugs Obtained By Stern Volmer Equation for Interaction with Cystatin

<table>
<thead>
<tr>
<th>Drug Parameter</th>
<th>( K_{sv} ) (Stern-volmer Constant)</th>
<th>( K ) (Binding constant)</th>
<th>( N ) (number of binding sites)</th>
<th>( \Delta G^o ) (Free energy change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HALOPERIDOL</td>
<td>( 15 \times 10^6 )</td>
<td>( 7.988 \times 10^6 )</td>
<td>1.1</td>
<td>-39.377</td>
</tr>
</tbody>
</table>

Absorption spectra of cystatin in the presence and absence of Haloperidol

The interaction between Haloperidol with cystatin was studied from UV-VIS absorption spectral data cystatin concentrations was fixed at 1µM while the haloperidol concentration was varied from 0.02µM-0.1µM. Absorption spectra of native cystatin and in presence of Haloperidol were recorded in the range of 200-300 nm. The UV absorption intensity of cystatin increased with the variation of Haloperidol concentration. UV absorbance spectra of cystatin, Haloperidol and their complexes are shown in [Fig-5] BC showed peak in the region 200-210 nm while on complexation with Haloperidol profound changes were introduced and there was peak shift of 30nm (red shift) in the range of 200-300 nm, with deep enhancement in absorbance. Shows that some complex formation might be taking place between haloperidol and cystatin[14,15]

**Figure 5:** UV-vis spectra of Cystatin in the presence and absence of Haloperidol

Cystatin concentrations was fixed at 1µM while the Haloperidol concentration was varied from 0.02µM-0.1µM. Absorption spectra of native BC in the presence and absence of Haloperidol were recorded in the range of 200-300 nm.

Inhibitory activity of Cystatin in the presence of Haloperidol

The obtained data indicates that the inactivation of Brain cystatin by Haloperidol is concentration dependent. 1µM cystatin was incubated with increasing concentrations of the drug (0.02-0.1µM) in 50 mM sodium phosphate buffer pH 7.5 at room temperature for 30 min and its inhibitory activity was determined by cascinolytic assay of papain [26]. The activity of native cystatin was taken as 100%. The exposure of 1µM of cystatin to varying concentrations of Haloperidol resulted in remarkable loss in its antiproteolytic activity. At concentration as low as 0.06µM haloperidol showed 42% loss in activity of cystatin. On interaction with 0.02 µM haloperidol, 29% loss of BC activity was noticed [Table-2]. At 0.1µM drug concentration the inhibitor retained only 36% of its original papain inhibition potential. For total protein fluorescence. The slits were set at 10 nm for excitation and emission. The path length of the sample was 1 cm.

**Table 2:** Inhibitory Activity of Cystatin In the Presence of Haloperidol

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Haloperidol concentrations with cystatin</th>
<th>% Remaining Inhibitory Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cystatin alone</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Cystatin + 0.02 µM</td>
<td>71 ± 0.938</td>
</tr>
<tr>
<td>3</td>
<td>Cystatin +0.04 µM Haloperidol</td>
<td>65 ± 0.915</td>
</tr>
<tr>
<td>4</td>
<td>Cystatin +0.06 µM Haloperidol</td>
<td>58 ± 0.920</td>
</tr>
<tr>
<td>5</td>
<td>Cystatin +0.08 µM Haloperidol</td>
<td>43 ± 0.770</td>
</tr>
<tr>
<td>6</td>
<td>Cystatin +0.1 µM Haloperidol</td>
<td>36 ± 0.911</td>
</tr>
</tbody>
</table>

Changes in the inhibitory activity of cystatin after incubation for 30 min with increasing concentration of Haloperidol. Cystatin (1µM) was treated with varying concentration of Haloperidol (0.02 µM-0.1µM) for 30min in the final reaction volume of 1 ml in 0.05M sodium phosphate buffer pH 7.5.
All data are expressed as mean ± S.E for four different sets of experiments. Statistical significance was conducted employing ONE WAY ANOVA. A probability level of 0.05 was selected showing results are significant.

4. Discussion

Haloperidol It is classified as a highly potent neuroleptic compound possessing a strong activity against delusions and hallucinations, most likely due to an effective dopaminergic receptor blockade in the mesocortex and the limbic system of the brain. The drug is rapidly absorbed. Plasma-levels reach their maximum within 20 minutes after injection. It is known that the distribution, free concentration and the metabolism of various drugs are strongly affected by drug–protein interactions in the blood stream [11,19,16]. This type of interaction can also influence the drug stability and toxicity during the chemotherapeutic process [11].

The Haloperidol - cystatin interaction was chosen as an example of analytical study because of its importance as a common antidepressant. Sulkowski et al. [6] investigated the binding interaction of methotexate with BSA by monitoring the changes in the fluorescence emission spectra of the protein in the presence of Methotexate. The results are very similar to our results in which binding of haloperidol with Cystatin has been studied. The binding constant (K) between haloperidol and Cystatin was found to be 7.988 x 10^6 showing strong binding affinity. Haloperidol-cystatin interaction gave one binding site with negative value of ΔG showing the reaction to be spontaneous (Table-1). The interactions between gemcitabine hydrochloride (GEM) and BSA also showed similar results [7]. The values of n for GEM-BSA interaction varied from 1.6 to 1.09.

The purified inhibitor was incubated with increasing concentrations of Haloperidol and its antiproteolytic potential was determined. Haloperidol diminished the activity of cystatin with maximum inactivation of the inhibitor at higher concentration within a short span of time (Table-2). At lower concentration of inhibitor inactivation was less significant. Considerable activity (~58%) was retained by cystatin at 0.06 µM of drug concentration. At maximum concentration of the drug (0.1 µM) brain cystatin retained 36% activity. Drug induced inactivation of proteins has been reported earlier for other proteins also like horse liver alcohol dehydrogenase activity which was affected differentially by various drugs. Few like barbital, caffeine and diazepam exerted no effect however clopramide, sulpride, morphine reduced the activity and phenytoin enhanced ADH activity [24]. Absence of any drug induced conformational change in cystatin suggest that inactivation of the inhibitor may be related to subtle changes in the conformation of the protein induced by the drugs. Similar ligand binding functional changes have been shown for isocitrate dehydrogenase [25].

In conclusion from this study binding of haloperidol with cystatin has been established which is expected to open new doors and new avenues in the screening and design of antidepressant drugs that may be of importance in modern medical research. The work shows the side effect of haloperidol which may be further explored for the accurate dose of effective drug. Since, the pharmaceutical firms need standardized screens for protein binding in the first step of new drug design, this kind of study of interaction between brain cystatin with haloperidol would be useful as a model system in pharmaceutical industry, life sciences and clinical medicine.

References


