EFFECTS OF SOME ANTITUBERCULOUS AND ANTI-LEPROTIC DRUGS ON CATHEPSINS B, H AND L.

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ABSTRACT

The cysteine proteinases like cathepsins B, L and H are main hydrolytic enzymes present in lysosomes and play an important role in intracellular protein degradation. Tuberculosis and leprosy, both are tissue- destructive diseases. Main drugs used in chemotherapy of these diseases may inhibit the main lysosomal cysteine proteinases i.e. cathepsins B, L and H released during tissue destruction and thus prevent the further destruction of tissue by these enzymes. So the aim of this study is to see the effect of antituberculous and antileprotic drugs on these proteolytic enzymes. The effect of commonly used antituberculous and antileprotic drugs was screened on the activities of purified brain lysosomal cysteine proteinases namely cathepsins B [EC 3.4.22.1], L [EC 3.4.22.15] and H [EC 3.4.22.16]. Among the antileprotic drugs, only clofazimine inhibited the enzymic activities whereas dapsone had no effect whatsoever. In antituberculous drugs, rifampicin was the most inhibitory while isoniazid had little inhibitory potency. Streptomycin and pyrazinamide did not effect the activities at all. As regards the mechanism of inhibition, clofazimine and isoniazid inhibited the enzymes in a non-competitive manner with K_i values of 0.25 mM and 5.0 mM for cathepsin B, 0.071 mM and 0.833 mM for cathepsin L and 1.513 mM and 0.885 mM for cathepsin H, While rifampicin could effect in a competitive manner with K_i values of 0.03 mM, 0.125 mM and 0.027 mM for cathepsin B, L and H respectively.

KEYWORDS:

Brain, Lysosomes, Cysteine proteinases, Cathepsins, Antituberculous drugs, Anti-leprotic drugs.

INTRODUCTION

Lysosomes, the intracellular organelles, discovered by deDuve et al (1) are involved in a wide variety of natural phenomena of fundamental biological importance. In general terms, the activity of lysosomes in these diverse processes is dependent on the digestive activity of the constituent acid-hydrolases, which are enclosed in a semipermeable membrane. A large number of substances (2) including inorganic metal ions, small organic molecules, organic macromolecules and cell metabolites are known to accumulate selectively within the lysosomes and are called lysosomotropic

agents. In addition to these, some micro-organisms are also known to invade lysosomes such as *Mycobacterium leprae* (2) and Reovirus (3). *Mycobacterium leprae* is the causal organism in leprosy. Some of the drugs like chloroquine (4) and streptomycin (5) are also found to accumulate in the lysosomes at a tremendous rate. The lysosomotropic agents alter the regulatory processes of lysosomes in many ways and result in some lysosomal storage diseases (2) like haemochromatosis, Wilson's disease, Pompe's disease and many more. Disturbances are also caused by rupturing the lysosomal membrane as in case of silicosis (6) and gout (7). Lysosomotropic agents can alter the intralysosomal milieu by interacting with the potent array of hydrolytic enzymes e.g. proteinases, esterases, lipases etc.

The cysteine proteinases like cathepsin B [EC 3.4.22.1], cathepsin L [EC 3.4.22.15] and cathepsin

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H [EC 3.4.22.16] are the main hydrolytic enzymes present in lysosomes and are known to play an important role in intra-cellular protein degradation (8). These cathepsins are also involved in many pathological disorders like periodontal disease (9), muscular dystrophy (10) arthritis (11) pancreatitis (12), Alzheimer's disease (13), asthma (14) and tumor growth and metastasis (15). In many of these diseases, lysosomal enzymes are found to be present in extracellular/extralyosomal environment. To our knowledge no attempt has ever been made to see the effects of antimicrobial agents on the activity of these enzymes. Hence in the present study, we have evaluated the effects of some antileprotic and antituberculous drugs on the most important lysosomal cysteine proteinases, namely cathepsins B, L and H purified from brain.

MATERIALS AND METHODS

Materials: Fast Garnet GBC, p-chloromercuribenzoic acid and α -N-benzoyl-D,L-arginine- β -naphthylamide (BANA) were procured from Sigma Chemicals Co., St. Louis, USA. Z-Phe-Arg-4-methoxy- β -naphthylamide (Z-Phe-Arg-4m β NA), Z-Arg-Arg-4-methoxy- β -naphthylamide (Z-Arg-Arg-4-m β NA) and Leu- β -naphthylamide (Leu- β NA) were purchased from Bachem Feinchemikalein AG, Switzerland. Rifampicin, streptomycin and isoniazid were received as gift samples from IPCA Laboratories Pvt. Ltd. Bombay. Pyrazinamide was supplied by LYKA Laboratories, Bombay. Antileprotic drugs, clofazimine and dapsone were obtained from Bengal Chemicals, Baroda and Pharmaceutical Ltd. Calcutta, respectively.

All other chemicals used in experiments were of highest purity available. Glass double-distilled water was used for making all kinds of buffers/solutions.

Purification and assay procedure of Cathepsins: Cathepsins B (16) and L (17) both from goat brain and cathepsin H (18) from bovine brain were purified to apparent homogeneity. The purification procedure broadly included the steps like preparation of brain acetone powder, homogenisation in a buffer of pH 5.5, acid-autolysis at pH 4.0, 30-70% (NH₄)₂SO₄ fractionation, molecular sieve chromatography on Sephadex G-100 column, organomercurial affinity chromatography, ion-exchange chromatography on CM-Sephadex C-50 and DEAE-Sephadex A-50 column. The homogeneities of these enzymes were established by electrophoresis at pH 8.4 (19) and

4.5 (20). The activities of cathepsins B, L and H were measured colorimetrically using chromogenic synthetic substrates Z-Arg-Arg-4m β NA, Z-Phe-Arg-4m β NA and Leu- β NA respectively. The activity unit has been expressed as number of micromoles of 4-methoxy- β -naphthylamine/ β -naphthylamine liberated per min per ml enzyme solution at 37°C.

Preparation of stock solutions of drugs: The stock solutions (0.2M) of dapsone, rifampicin, isoniazid and clofazimine were prepared in dimethylsulfoxide (DMSO) and that of streptomycin in water. The stock solution (0.2M) of pyrazinamide was prepared in ethanol.

Effect of different drugs on the activities of cathepsins: The purified cathepsins were first activated with 2 mM cysteine at their pH optima (pH optima for cathepsin B (16) is 6.0, for cathepsin L (17) is 6.0 and for cathepsin H (18) is 7.0), separately for 10 min at 37°C. The 50 μ l of stock solution of different drugs under study were added separately to the activated enzyme assay mixtures (total volume 2.0 ml) to get the 5 mM final drug concentration. The resulting mixtures were incubated further for 10 min at 37°C separately. Afterwards the residual activities in each sample were estimated by the usual assay procedure (16-18). In control experiments, the equivalent amount of respective solvents were added to avoid any effect of the solvent whatsoever on the enzyme activities and percent residual activities were calculated with reference to control.

Effect of different concentrations of drugs on enzymes activities: The inhibitory drugs: clofazimine, rifampicin and isoniazid were further screened for their effect on these enzymes at different concentrations. Final concentrations of clofazimine for cathepsin B were 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mM, for cathepsin L were 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0 mM and for cathepsin H were 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mM. Similarly, the final concentrations of rifampicin taken for cathepsin B were 0.25, 0.5, 0.75, 1.0 and 2.0 mM, for cathepsin L were 0.1, 0.2, 0.3 and 0.5 mM and for cathepsin H were 0.1, 0.5 and 1.0 mM. Likewise, the concentrations at which the effect of isoniazid was observed were 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mM for cathepsin B; 0.25, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mM for cathepsin L and 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mM for cathepsin H. The enzymes were incubated with the above mentioned drug concentrations for 10 min at 37°C. Then, the residual

enzymic activities were measured by the usual assay procedures. The control experiments were also included where no drug was added.

Kinetic studies: The mechanisms of inhibition of cathepsins B, L and H by the above studied inhibitory drugs were also determined. In order to do so, the enzymatic activities were measured with varying concentrations of their respective substrates (Z-Arg-Arg-4mβNA for cathepsin B, Z-Phe-Arg-4mβNA for cathepsin L and Leu-βNA for cathepsin H) in presence and absence of fixed concentration of drugs. The inhibition constant, K_i and the type of inhibition caused by a particular drug was determined by a graph obtained by plotting reciprocals of substrate concentrations against reciprocals of enzyme activities (Lineweaver-Burk Plot) in presence and absence of drugs.

RESULTS AND DISCUSSION

Leprosy, caused by *Mycobacterium leprae* is a chronic granulomatous infection. Conventionally, all the forms of leprosy i.e. multibacillary or paucibacillary including both the polar types i.e. lapromatous and tuberculoid have been treated with dapsone alone. It has been established that dapsone, chemically related to sulfonamides antagonises PABA and inhibits bacterial folic acid. Though, dapsone is a first line chemotherapeutic agent used against leprosy, emergence of dapsone resistance has threatened the efficacy of monotherapy (upto 20% patients have relapsed). To deal with dapsone resistant strains of *M. leprae*, multidrug therapy (MDT) with clofazimine has been advocated. Sometimes rifampicin is also included in MDT and appears to shorten the duration of treatment. Since, the causal organism of leprosy i.e. *M. leprae* resides in the lysosomes (2), the storehouse of lysosomal cysteine proteinases, the present study was undertaken, to ascertain the effect of antileprotic drugs on these enzymes. Brain cathepsins were used because they are lysosomal in nature and are similar in properties with cathepsins from other tissues. Since the methods for purification of these cathepsins from brain have been perfected in our laboratory, their availability was easier.

Table I depicts the effects of antileprotic drugs (clofazimine and dapsone) alongwith rifampicin (primarily used as antituberculous drug) on the activities of lysosomal cysteine proteinases i.e. cathepsins B, L and H obtained from brain. The

effect was first observed at 5 mM final drug concentrations and it was found that dapsone does not inhibit these enzymes, whereas clofazimine, at this concentration caused complete inhibition. After establishing the inhibitory nature of clofazimine, it was thought proper to observe the effect of different concentrations of this drug on the activities of these cathepsins. From the results (Fig 1) it is quite clear that at 2 mM final concentration of clofazimine, cathepsin L had no residual activity, cathepsin B exhibited 40% activity while cathepsin H could retain only 20% activity. The inhibitory potency of this drug can be explained on the basis of structure-activity relationship. The main ring in clofazimine is analogous to oxidized flavin ring which may modify the reduced thiol group ($-CH_2SH$) present at the active-site of these enzymes. Dapsone does not possess this type of structural feature and hence is not inhibitory to these cathepsins. These result correlate well with the inflammatory reactions observed during leprosy treatment when dapsone is used as the sole therapeutic agent. When clofazimine is included in the treatment such inflammatory reactions are not observed because the inflammation causative agents i.e. lysosomal hydrolytic enzymes are inhibited by clofazimine. It has been reported that monotherapy with dapsone for both tuberculoid and lepromatous type of infections, results into reversal reaction and lepra reaction (21) respectively. The reversal reaction is a manifestation of delayed hyper-sensitivity of *M. leprae* antigen whereas, lepra reaction is due to release of antigen from killed bacilli. Lepra reaction (22) may be mild, severe or life-threatening (erythema nodosum leprosum). Both these side reactions are treated with the administration of clofazimine, probably because of the anti-inflammatory property of the drug. The inhibition of cathepsins B, H and L, which certainly are involved in many inflammatory conditions (11, 12) has some clinical significance for controlling the side reactions caused by non-inhibitory dapsone. Clofazimine inhibited these enzymes in a noncompetitive manner as K_m value remained constant while V_{max} value decreased. The values of inhibition constant K_i are shown in Table II.

Table I also shows the effects of commonly used first line antituberculous drugs viz., rifampicin, isoniazid, pyrazinamide and streptomycin on these cathepsins. Rifampicin and isoniazid were found to be inhibitory to these cathepsins (Table I). In contrast streptomycin and pyrazinamide had no effect

whatsoever on the enzyme activities. The activities of these enzymes i.e. cathepsins B, H and L were also determined in the presence of different concentrations of rifampicin (Fig. 2) and isoniazid (Fig. 3). Rifampicin completely inhibited cathepsin L at 0.5 mM, cathepsin H at 1 mM and cathepsin B at 2 mM final concentrations. Isoniazid behaved in a different manner with cathepsin B which showed only 30 % inhibition at 5 mM concentration. But in case of cathepsin H and L it showed complete inhibition at this concentration. The extent of enzymic inhibition by rifampicin was more than that of isoniazid among these drugs. (Fig.2 and Fig.3). Any conclusion for the inhibition of these cathepsins by rifampicin and isoniazid on the basis of structure-activity relationship, could not be drawn at this juncture. It is well known fact that tuberculosis is a tissue destructing disease which progresses by means of ulceration and caesation etc. Hence, the role of lysosomes and their battery of hydrolytic enzymes can rightly be suspected in such diseases which involve breakdown of tissue by the action of hydrolytic enzymes. Moreover, lysosomal cysteine proteinases when secreted can be very harmful for their environment resulting in pathological conditions. As the main lysosomal cysteine proteinases i.e. cathepsins B, L and H are well inhibited by rifampicin and isoniazid and not by streptomycin and pyrazinamide, probably this makes the combination of rifampicin-isoniazid more effective in curing tuberculosis, a dreaded disease. It may be mentioned here that conventional regimens for tuberculosis consist of isoniazid and streptomycin (23). Optimum results require 12-18 months therapy with <2-4 % relapse. Under routine field treatment conditions early abatement of symptoms and long duration therapy, only 25-30 % patients complete the 18 months course. To overcome these difficulties short-term regimens consisting of combination therapy with isoniazid and rifampicin were tried which yielded better results after 6-9 months therapy (23-25). Lastly, the mechanism of inhibition of these cathepsins by

rifampicin and isoniazid was also determined (Inhibition constant, K_i values shown in Table II). When the activities of cathepsins B, L and H were measured in presence of isoniazid, the V_{max} values were decreased and the K_m values remained constant (data was analysed by Lineweaver-Burk plot). This showed that the drug is the non-competitive inhibitor of these cathepsins. In case of rifampicin, the V_{max} values remained constant and K_m values increased, proving it to be a competitive inhibitor of these cathepsins. Since, the combination of drugs that inhibit lysosomal cathepsins very strongly shows faster recovery, suggest some role of these enzymes in aggravating the diseased conditions. However, more elaborate studies are required to reach any concrete conclusion. The effect of antileprotic and antituberculous drugs on cathepsins B, H and L is being reported here for the first time. Earlier the effects of some steroidal and non-steroidal anti-inflammatory drugs on cathepsin L (26) have been studied in our laboratory.

Although, the present study may not clearly indicate the mechanism of action of these drugs vis-à-vis these cysteine proteinases, yet clofazimine has been established as a potent inhibitor of cathepsins B, H and L. The short-term regimens for the tuberculosis are suggestive of the involvement of these enzymes in this tissue destructive disease.

It can thus be concluded that the main drugs used in chemotherapy of leprosy and tuberculosis inhibit the lysosomal cysteine proteinases i.e. cathepsins B, L and H released during the tissue destruction and thus prevent the further destruction of tissue by these enzymes.

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Table 1

Effect of various antileprotic and antituberculous drugs on cathepsins B, L and H activities at 5mM drug concentration.

Drug	Residual activity (%)		
	Cathepsin B	Cathepsin L	Cathepsin H
ANTILEPROTIC			
Clofazimine	0.00	0.00	10.0 ± 1.5
Dapsone	99.6 ± 0.2	99.3 ± 0.4	95.3 ± 1.2
ANTITUBERCULOUS			
Pyrazinamide	99.5 ± 0.3	99.8 ± 0.2	91.4 ± 1.6
Streptomycin	98.5 ± 1.1	99.7 ± 0.1	98.1 ± 1.3
Rifampicin	0.00	0.00	0.00
Isoniazid	70.0 ± 2.1	0.00	0.00

The enzyme, cathepsins B, L and H were incubated at 37°C with 5mM final drug concentration for 10 min., separately. The percent residual activities were calculated with respect to control experiments. The results are mean ± S.D. of three different experiments.

Table 2

Inhibition constant, K_i values and V_{max} values for cathepsins, B, L and H

Drug	K_i (mM) of cathepsins			V_{max} (units/mg) of cathepsins			Type of inhibition
	B	L	H	B	L	H	
Clofazimine	0.25	0.071	1.513	18.2	8.1	0.177	Non-competitive
Rifampicin	0.03	0.125	0.027	40.0	19.04	0.294	Competitive
Isoniazid	5.0	0.833	0.885	26.2	6.76	0.138	Non-competitive

The data were analysed by Lineweaver-Burk plot. The K_m values of cathepsin B, H and L under experimental conditions with Z-Arg-Arg-4mβNA, Leu-βNA and Z-Phe-Arg-4mβNA were found to be 0.286 mM, 0.290 mM and 0.067 mM, respectively. The V_{max} values in absence of any drug has been calculated as 40.0, 19.04, 0.294 units/mg for cathepsins B, L and H, respectively.

Fig.1. Effect of clofazimine on cathepsin B, H and L: Clofazimine is most inhibitory to cathepsin L. Half maximal activity for the enzyme is lost at 0.25 mM drug concentration, whereas for cathepsin H and B, it is 0.75 mM and 1.5 mM, respectively.

Fig.2. Effect of rifampicin on cathepsin B, H and L: Cathepsin H and L are most sensitive toward rifampicin inhibition. The drug exhibits a parallel inhibitory effect on these enzymes till ~ 85 % inhibition is achieved. Half maximal activity of cathepsin L, H and B is inhibited at 0.1 mM, 0.2 mM and 0.8 mM rifampicin concentration, respectively

Fig.3. Effect of isoniazid on cathepsin B, H and L: Isoniazid is least inhibitory to cathepsin B. It inhibited ~ 50 % activity of cathepsin L and cathepsin H at 0.55 mM and 0.9 mM, respectively, whereas, similar level of inhibition for cathepsin B could not be achieved even at 5 mM final drug concentration.

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