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SHORT COMMUNICATION

INHIBITION OF HUMAN SYNOVIAL β-GLUCURONIDASE BY STEROIDAL COMPOUNDS

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TO FOLLOW

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INTRODUCTION

In our laboratory we have focused on several enzymes, among them β-glucuronidase, which have been shown to be released in abnormal quantities during chronic inflammatory diseases such as Rheumatoid Arthritis.[1,2] This enzyme, which is involved in the catalysis of β-glucuronides, has been characterized in mammalian tissues.[3] In previous studies[4,5] we have demonstrated that it was inhibitied by a number of synthetic antiinflammatory gold (I) complexes, such as gold (I) thiomalate (Myochrisin) and gold (I) thiosulfate (Solganol). although the mechanism of inhibition has not been verified we have suggested from these and previous studies[6] that a thiol group on the enzyme may be the primary site of binding to the gold complex.

Since steroids have been one of the preferred choices of drugs as antiinflammatory agents[7,8] it was of interest to us to determine if they inhibited the enzyme and the structural features important in the inhibitory process. A number of commonly used steroids were tested, i.e., cortisone and hydrocortisone, as well as several newly synthesized compounds, 24,25 dihydrolanosterol-24-oxime, 25-aminolanesterol and 6-nitrocholesterol. The latter three compounds are slightly aqueous soluble, and one of them has a nitrogen group which we anticipated would increase its binding to the enzyme. 6-Nitrocholesterol may be shown to have some potential as an antiinflammatory drug.

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EXPERIMENTAL

Materials

Human synovial fluid was obtained from the Hughston sports Hospital and the Medical Center & Doctors Hospital of Columbus, Georgia and from the Medical Center of East Alabama of Opelika, Alabama. Phenolphthaleinglucuronic acid, tris buffer, glycine ammonium sulfate, N,N,N'N'-tetramethylenediamine (EDTA), cortisone and hydrocortisone were purchased from Sigma. CM-cellulose and DEAE-cellulose were purchased from Whatman, acrylamide ad N,N'-methylene bisacrylamide from Serva, and protein assay kits from Bio-Rad. All other chemicals were of reagent grade.

METHODS

Chemical synthesis of 25-aminolanosterol[9,10] and 6-nitrocholesterol[11,12] have been described elsewhere. 24,25-Dihydrolanosterol-24-oxime was prepared from 24-ketolanosterol[13] by established procedures.[14] The structure and purity (>98%) of each steroid was determined as previously described.[15]

Purification of β-glucuronidase was carried using a modification[16] of previously employed methods. Ammonium sulfate, DEAE-cellulose and CM-cellulose fractionation were utilized. Enzyme activity was measured by a Fishman modification of the method of Talalay[17] at pH 4.5 in 0.1 M acetate buffer, and the resulting chromophore was measured at 540 nm, pH 10.8, on a Gilford Model 250 Recording Spectrophotometer. Protein concentrations were deterined using the Bio-Rad assay.[18]

Enzyme inhibition was carried out as follows. Stock solutions of each steroid were prepared in either absolute ethanol or dimethyl sulfoxide. Working solutions were prepared by dissolving an aliquot of the stock solution in phosphate buffer which was then brought to 10 mM at pH 7.0. Controls were run along with the tests and were prepared in the same manner with the omission of the steroid. Inhibition took place by incubation of the enzyme (0.25 μM) with various aliquots of the steroid working solution (0.1–2.0 mM) at 25°C and allowing the reaction to proceed for at least 6 h, or until inhibition reached a steady-state. Samples (100 μL) were then removed and their activity recorded.

RESULTS AND DISCUSSION

Inhibition of the enzyme by all the steroids followed a time-dependent pathway, reaching a steady-state after 6 h. Measurement of the remaining enzyme activity was determined using substrate concentrations ranging from 10 μM to 100 μM. From these data Lineweaver-Burk, double-reciprocal plots were constructed. The results of the cortisone inhibition study are presented in Figure 1, while the inhibition constant (K_i) is given in Table 1. The curves indicate that cortisone is acting as an uncompetitive inhibitor of the enzyme. Similar results were obtained with 17-hydrocortisone, 24,25-dihydrolanosterol-24-oxime and 25-aminolanosterol. The inhibition constants for each of these inhibitors are also presented in Table 1 and their range was found to be between 352 μM and 508 μM. However, the results of the studies using 6-nitrocholesterol as the inhibitor suggested a different mechanism.

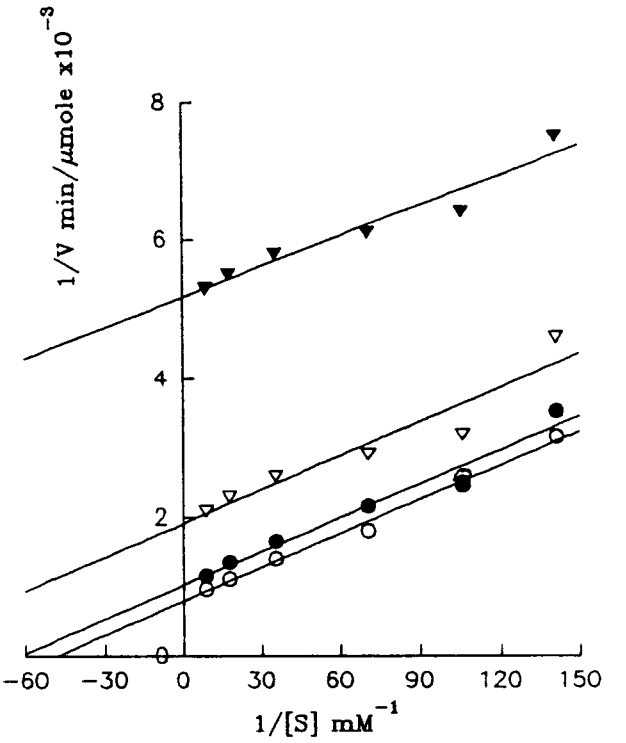

Figure 1 Lineweaver-Burk Plots of β-Glucuronidase Inhibition by Cortisone. The concentrations of the steroid are; –○–, 0 mM (control), –●–, 0.1 mM, –▽–, 0.5 mM and –▼–, 2.0 mM. The enzyme-steroid compounds were incubated in 0.1 M phosphate buffer, pH 7.0 and 25°C for 6 h. The activities were measured after 24 h incubation at 37°C in 0.1 M acetate buffer, pH 4.5. Each experiment has been run in triplicate.

Table 1 Inhibition constants (K_i) and the type of inhibution obtained for steroid inhibition of β-glucuronidase

Steroid	K_i(μM)	Type of inhibition
Cortisone	352	Uncompetitive
Hydrocortisone	375	Uncompetitive
24,25-Dihydrolanosterol-24-Oxime	437	Uncompetitive
25-Aminolanosterol	508	Uncompetitive
6-Nitrocholesterol	180	Mixed competition

The curves presented in Figure 2 indicate that this steroid is acting as a mixed competitive inhibitor, and the K_i of 181 μM (Table 1) suggests that it binds somewhat more strongly to the enzyme.

Since the primary modifications in these steroids (other than the nitro compound) take place in the side-chain extention from C-17 of ring D or at C-11 in ring C it appears that the influence of those rings or any projections from ring D have little involvement in the inhibition of the enzyme. We propose that there is little or no

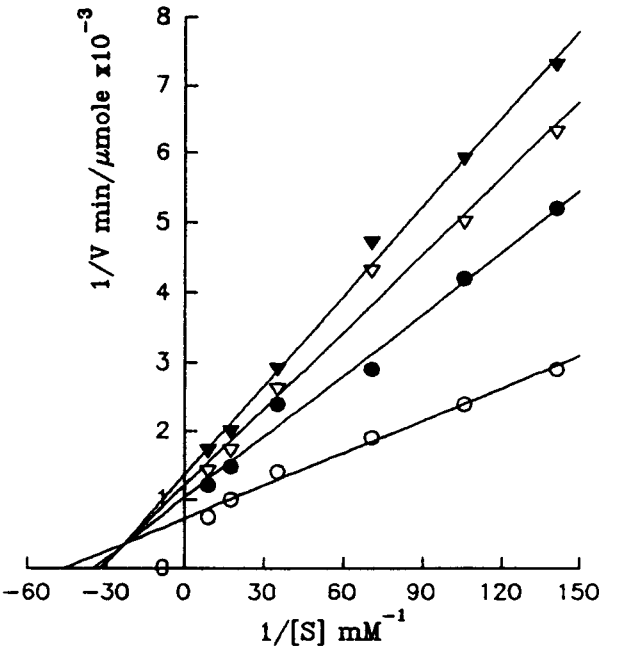

Figure 2 Lineweaver-Burk Plots of β-Glucuronidase Inhibition by 6-Nitrocholesterol. The concentrations of the steroid are: -○-, 0 mM, -●-, 0.1 mM, -▽-, 0.5 mM and -▼-, 2.0 mM. Experimental conditions are given in Figure 1.

binding of these rings to the enzyme, or whatever binding occurs provides little influence on the kinetics of the inhibition. However, inhibition by the 6-nitrocholesterol derivative is not only significantly greater ($K_i = 181\ \mu M$), but it follows a mixed competition profile (Figure 2). This evidence suggests that the steroids most likely bind to the enzyme at rings A and B and the type of substituted group(s) on those rings could be of significance. This view may be supported by the fact that the nitro group is the only extension from ring B. It may also be observed that all the steroid derivatives have an oxygen at position C.3 in ring A, and while that probably provides a binding site for inhibition it is not responsible for differentiating the steroids behavior toward the enzyme. It is possible that the NO_2 group, being highly polar, may bind to an active site group leaving to a different type of inhibition from that of tye other steroid derivatives. At the same time its location at position C-6 may also be of significance.

It now appears that an existing function or a substituent on rings A and B of the steroids, can effect the type of inhibition of the enzyme. Other steroids are being prepared, as well as the isolation and purification of other synovial enzymes, in order to test this hypothesis.

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