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COMPARATIVE INHIBITION OF HUMAN ALKALINE PHOSPHATASE AND DIAMINE OXIDASE BY BROMO-LEVAMISOLE, CIMETIDINE AND VARIOUS DERIVATIVES

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Abstract—Analogues of bromo-levamisole and guanidine derivatives including cimetidine are examined *in vitro* in order to investigate their comparative inhibition, towards alkaline phosphatase (ALP) from human liver and diamine-oxidase (DAO) from human placenta. Bromo-levamisole, considered as a potent selective uncompetitive inhibitor of ALP (K_i, $2.8 \cdot 10^{-6}$ M at pH 10.5) is shown to be a non-competitive inhibitor of DAO ($K_i = 7 \cdot 10^{-4}$ M). According to the structure–inhibition relationship, the imidazole ring is important for ALP and DAO inhibition. The phenyl ring of bromo-levamisole is required for ALP inhibition but not for DAO inhibition, which is mediated mainly by aminoguanidine or guanidine groups. These results have allowed the selection of cimetidine, an H_2-antagonist but also an immunomodulating compound, as inhibitor of these two enzymes. Cimetidine is an uncompetitive inhibitor of ALP ($K_i = 3.2 \cdot 10^{-3}$ M at pH 10.5), and a good inhibitor of DAO ($I_{50} = 3.8 \cdot 10^{-4}$ M). The K_i of ALP is commonly calculated at pH 10.5, but to study the role of the enzyme at the physiological pH, the inhibition has also been performed at pH 7.4. The K_i values are only slightly affected by this pH variation. So far several compounds, including levamisole, imidazole, theophylline and aminoguanidine are known to possess immunomodulating activities *in vivo* and/or *in vitro* and inhibit ALP and/or DAO. Therefore, it seems reasonable to assume that the inhibition of enzymes is involved in the immunomodulating effects of these drugs, when the ranges of active concentrations are similar for these properties.

In pharmacology, when a drug is tested *in vivo* it is usual to seek a target in an organism to explain the biological effect.

The pharmacological properties of the drug are in many instances directly or indirectly linked to the enzyme inhibition.

Levamisole, for instance, is a very interesting drug which has been shown to inhibit three different enzymes. The succinate deshydrogenase (SDH 1.3.99.1) from nematode [1], the diamine oxidase (DAO EC 1.4.3.6) from human placenta [2] and alkaline phosphatase (ALP EC 3.1.3.1) from human [3], murine [4] and canine origin [5].

The anthelmintic properties of levamisole are linked to the inhibition of SDH, producing muscular paralysis of nematode, but the decrease of sperm motility by this drug is not clearly related to DAO inhibition. Moreover the immunopotentiating effect of levamisole until now has not been related to enzyme inhibition.

Nevertheless, recent advances concerning the role of ALP and DAO in cellular regulation processes should make it possible to correlate the immunomodulating effect to enzyme inhibition. It has been claimed that mammalian ALP dephosphorylates a variety of proteins containing phosphotyrosine residues [6] and that levamisole increases the autophosphorylation of membrane proteins from human liver [7]. As the regulation of many metabolic processes occurs by phosphorylation (phosphoprotein kinase) and dephosphorylation (phosphoprotein phosphatase), the ALP may be involved in cellular regulation by this mechanism.

DAO, unevenly distributed in the organism, is involved in the terminal catabolism of polyamines. Most of the early interest in DAO stemmed from the high enzyme activity found in physiological barriers, such as the placenta or intestinal mucosa, where it protects against a high concentration of putrescine and other polyamines [8]. The presence of polyamines amplifies the proliferative response of lymphocytes *in vitro* [9]. Similar effects of aminoguanidine, a potent inhibitor of DAO, suggest an active role of endogenous polyamines in proliferative events [10]. Keeping these possibilities in mind, we describe in this paper, the results of structure–inhibition studies of DAO and ALP by various compounds. The goal of the structure–inhibition relationship is to select compounds with immunoenhancing activity.

Several structures have been selected for this purpose (Fig. 1).

Compounds considered as inhibitors of ALP and DAO. (−)6-(4-Bromophenyl)2,3,5,6-tetrahydro imidazo [2,1-b] thiazole or bromo-levamisole (A) [2, 11], imidazole (J) [12, 13], L-homoarginine (K) [14, 15], aminoguanidine (L) [10], theophylline (N) [16].

Compounds tested and selected as intermediates of synthesis of levamisole. 2,3,5,6-Tetrahydroimidazo [2,1-b] thiazole (B), 4-phenylimidazole (C), 2-thioimidazolidine (E), (±)2-thio 4-phenylimidazolidine

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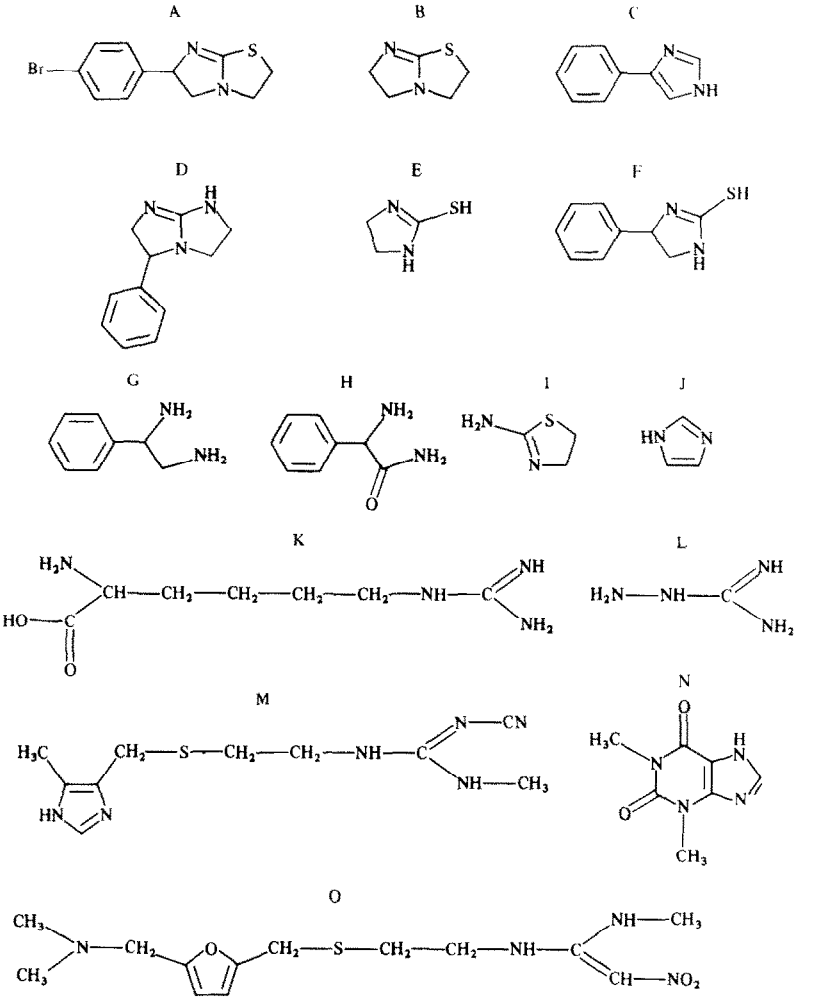

Fig. 1. Selected compounds for inhibition of human liver alkaline phosphatase and human placenta diamine oxidase.

(F), (±) β-amino phenethylamine (G), (±) phenylglycinamide (H), 2-amino 2-thiazoline (I).

Compounds selected as potential inhibitors as ALP and DAO. Imafen (D), cimetidine (M), ranitidine (O). Compound D is structurally related to compound A, except for the position of the phenyl ring, and the substitution of the S atom by an N atom. The compounds M and O are structurally related to compound K.

MATERIALS AND METHODS

Chemicals. 1,4[^{14}C]putrescine (specific activity 90 Ci/mole) was purchased from CEA (France). Bromo-levamisole, L-homoarginine, 4-phenylimidazole, imafen, 2-thioimidazolidine and 2-amino 2-thiazoline were purchased from Janssen Pharmaceutica, Division Aldrich-Europe B-2340 (Belgium). Aminoguanidine, 3-[morpholino] propane sulfonic acid (MOPS) and theophylline were purchased from Sigma Chemical Co (St Louis, MO). Imidazole came from Prolabo (Paris, France), and 2 (*tert*-butylphenyl) 5-(4-biphenyl) 1,3,4-oxadiazole (butyl-PBD), from Fluka, AG (CH-9470 Buchs, Switzerland). Ranitidine and cimetidine were obtained from Fournier S.A. Laboratories (Dijon, France) and Smith Kline and French Laboratories (Paris, France), respectively.

(±) β-Amino phenethylamine dihydrochloride (compound G) was synthesized by the following procedure: a suspension of 16.8 g of phenylglycinonitrile, HCl (0.1 moles) in 150 ml of alcoholic hydrogen chloride (1.5 N) was cooled in a Parr shaker bottle. Then 0.75 g of platinum oxide was carefully added while the temperature was kept below 10° and the mixture hydrogenated under 40 kg of H_2. After stirring for 24 hr, the platine was eliminated and compound G obtained by filtration as a white powder of dihydrochloride. Yield 66%, m.p.: >265° (lit. 285° [17]). Elementary analysis and spectroscopic data (^{1}H-NMR) were consistent with assigned structure.

(±) Phenylglycinamide hydrochloride (compound H) was prepared according to the following procedure: a suspension of 25 g of phenylglycinonitrile, HCl (0.15 moles) in 300 ml of etha-

nolic hydrogen chloride (5 N) was stirred overnight at room temperature. The precipitated product was collected, washed with ether and dried to obtain 16 g (60%) of compound H as a white powder; m.p. > 265° (lit. 272° [18]). Elementary analysis and spectroscopic data (^{1}H-NMR) were consistent with assigned structure.

(±) 2-Thio 4-phenylimidazolidine (compound F) was synthesized according to the following procedure: from 5 g of compound G (0.024 moles) the free base was liberated as follows; the compound G was dissolved in 50 ml of NaOH (1 N) and the solution extracted three times with 400 ml of ether. 2.5 g of carbon disulfide (0.033 moles) were added dropwise to 2 g of (±) β-amino phenethylamine (0.014 moles) in 100 ml absolute ethanol (exothermic reaction). The solution was stirred and refluxed for 5 hr. The mixture was then alkalized with sodium hydroxide and the compound F was extracted with ethylacetate to obtain 1.7 g (65%) of a white powder, m.p. 193° (lit. 195° [19]). Elementary analysis and spectroscopic data (^{1}H-NMR, ^{13}C-NMR) were consistent with assigned structure.

2,3,5,6-Tetrahydro imidazo [2,1-b] thiazole hydrochloride (compound B) was prepared as follows: a mixture of 12.3 g of 2-thioimidazolidine (0.12 moles) and 20% aqueous KOH in 2-propanol (350 ml) was added dropwise to a stirred and refluxing mixture of 1,2-dibromoethane (51 g, 0.27 moles) and 23.4 g of $NaHCO_3$ (0.27 moles) in 2-propanol (180 ml). The refluxing continued for 7 hr and the mixture was filtered. The solvent was completely evaporated under reduced pressure and the residue was treated with a KOH solution and the base extracted with toluene. The tetrahydro imidazo [2,1-b] thiazole was purified by chromatography ($SiO_2/CHCl_3$) or by distillation (82°, 0.4 mmHg) and was converted into hydrochloride, yield 58%, m.p. 181°. Elementary analysis and spectroscopic data (^{1}H-NMR, ^{13}C-NMR, MS) were consistent with assigned structure.

Determination of K_i *and* I_{50}. For K_i determination, inhibitors were dissolved in buffer and measurements made at five different substrate concentrations.

The Michaelis equation was fitted directly to untransformed data by non-linear least squares regression according to the Cleland's iterative method [20]. K_i value and standard error were obtained by replotting $1/V'_m$ versus the concentration of the inhibitor. For I_{50} evaluation, the procedure of Witwicki and Chidambaram was used [21]. The concentration of inhibitor at which 50% inhibition occurs was calculated for a linear regression curve, $1/V$ versus the concentration of the inhibitor.

ALP purification. Human liver ALP was partially purified using butanolic extraction and ammonium sulfate fractionation as described [22]. Chromatography (D.E.A.E. Sephadex A 50) was then carried out. The active fractions, after pooling, were concentrated by pressure filtration through Diaflo membranes (Amicon). The final preparation contains the following specific activities (IU/mg protein): alkaline phosphatase, 1.2; acid phosphatase, 0.013; 5′-nucleotidase, 0.004.

One international unit (IU) corresponds to one μmole of substrate hydrolysed per minute.

ALP activity assay. The final concentrations in the test for ALP measurement were: glycine, pH 10.5, 100 mM or 3-[morpholino] propane sulfonic acid (MOPS), 50 mM, pH 7.4; $MgSO_4$, 1 mM; *p*-nitrophenyl phosphate 16 mM at pH 10.5 or 50 mM at pH 7.5.

p-Nitrophenol formation was recorded at 405 nm, at 30°, using a Gilford spectrophotometer.

DAO-purification. Given the high concentration of DAO in amniotic fluid, the latter was only purified by centrifugation to remove cells. Specific activity = 0.1 IU/mg protein.

DAO activity assay. DAO activity was assayed by the method of Okuyama *et al.* [23] as modified by Kusche *et al.* [24] which measures Δ1[^{14}C]pyrroline formed from [^{14}C]putrescine. The assay mixture contained 0.1 ml of putrescine $0.25 \cdot 10^{-3}$ M (specific activity 1 μCi/ml), 0.1 ml of inhibitor, 0.2 ml of amniotic fluid plus 0.1 ml of phosphate buffer, 0.2 M pH 7.4.

After 15 min at 55°, the DAO reaction was stopped with 1 ml of aminoguanidine 10^{-2} M in Na_2CO_3 2%, pH 10. Extraction of the product was performed with 10 ml of scintillating fluid (butyl-PBD 5‰ in toluene). After freezing at −20° for 15 min, the toluene phase was easily collected and counted with 93 ± 2% efficiency.

Assays blanks were carried out without enzyme. Two tests were performed for each inhibitor concentration.

RESULTS AND DISCUSSION

Inhibition of ALP

The most striking feature of ALP is its optimum alkaline pH, but the significance of this pH effect *in vivo* has never been clear. However, the inhibition at physiological pH is considered more meaningful.

In this respect it is interesting to emphasize that the K_i values are only slightly affected by pH variations from 10.5 to 7.5 (Table 1). The order of potency according to the K_i values is:

$$A >>> N > B, M, K > J > H > I.$$

This falls in line with earlier data showing the inhibition by compounds A [11], J [12], K [14, 15] and N [16].

Bromo-levamisole (compound A) is the most potent inhibitor of liver ALP isoenzyme, $K_i = 2 \cdot 10^{-6}$ M pH = 7.4.

According to Fishman [25], compound K is the most potent inhibitor among amino-acids but the most original result in our study is the inhibition of ALP by cimetidine (compound M), $K_i = 1.8 \cdot 10^{-3}$ M at pH = 7.4, selected for its anti-H_2 effect.

A marked difference is observed between compound A and compounds H, I and J which are poor inhibitors with a $K_i > 10^{-2}$ M.

Structure–inhibition relationship of ALP

The stereospecificity of liver ALP inhibition is a feature of compounds A [11] and K [14] (dextrogyre derivatives are inactive). Aminoacid group of compound K and parabromophenyl ring of compound A are directly involved in this stereospecificity.

To investigate the role of the phenyl ring, analogue

Table 1. Inhibition of alkaline phosphatase from human liver and diamine oxidase from human placenta by selected compounds

Compound	Alkaline phosphatase $K_i \pm$ SD (mM)		Diamine oxidase $I_{50} \pm$ SD (mM)
	pH = 10.5	pH = 7.4	pH = 7.4
A	0.0028 ± 0.0003	0.0052 ± 0.0006	0.70 ± 0.09
	UC	UC	NC
B	1.2 ± 0.1 UC	3.7 ± 0.4 UC	0.72 ± 0.08
C	NI	NI	1.2 ± 0.1
D	NI	NI	3.2 ± 0.4
E	NI	NI	NI
F	NI	NI	18 ± 2
G	NI	NI	23 ± 3
H	24 ± 3 UC	50 ± 5 UC	18 ± 2
I	60 ± 5 UC	ND	2.1 ± 0.2
J	14 ± 1 UC	ND	5.5 ± 0.7
K	4.3 ± 0.4 UC	5.4 ± 0.6*	12 ± 2
L	NI	NI	0.0040 ± 0.0005
M	3.2 ± 0.4 UC	1.8 ± 0.2 UC	0.38 ± 0.08
N	0.60 ± 0.04	0.82 ± 0.09 UC	NI
O	NI	NI	NI

SD, standard deviation.
NI, 10% inhibition at 10^{-2} M.
ND, not determined.
UC, uncompetitive inhibition.
NC, non-competitive inhibition.
*, in glycine buffer.

B has been prepared. This compound has only one five-hundredth of the potency of the analogue A. These findings support the hypothesis that the hydrophobic phenyl ring and stereoisomery are closely involved in the tight binding of the A analogue.

Among the following mixed derivatives, tested compounds H, I and J cause moderate inhibition of ALP, and compounds C, D, E, F and G have no effect. Therefore, the tetrahydro imidazo [2,1-b]thiazole heterocycle, stereoisomery and phenyl ring must be preserved to keep a high inhibition. Recent developments in the substitution of this heterocycle [26] should take these results into account, because many syntheses in organic chemistry are carried out without taking into account the nature of the receptor of the drug.

We note that compounds M and O, selected as anti-H_2 drugs, exhibit very different behaviour towards ALP. As the O derivative has no effect, the imidazole ring of compound M might be involved in the inhibition because guanidine alone is not an inhibitor (data not shown). Furthermore, the effect of compound M is fivefold that of compound J.

These results confirm the importance of the chain of methylene units, as verified for compound K, which is more potent than L-arginine [25]. Curiously, the differences between compounds M and O are similar to the inhibition of hepatic microsomal system by M but not by O [27].

Nature of ALP inhibition

All the compounds studied have an inhibition of uncompetitive nature as indicated by Hanes plots (Fig. 2). These results, previously reported for compounds A [11], K [14] and N [16], are consistent with the presumed action of inhibitor in stabilizing the phosphoryl–enzyme or the phosphate–enzyme complex [28]. Nevertheless the main difficulty is to correlate structure with the proposed mechanism of inhibition, which has not yet been specified.

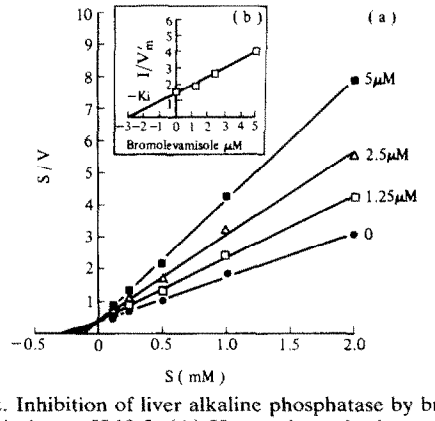

Fig. 2. Inhibition of liver alkaline phosphatase by bromolevamisole at pH 10.5. (A) Hanes plots of substrate concentration (S) divided by initial velocity (V) versus substrate concentration (S) at different concentrations of bromo-levamisole. (B) Determination of K_i. (The error bars are smaller than the symbols used to represent the experimental points.)

It should be noted that the uncompetitive inhibitors of ALP are more specific for this enzyme than

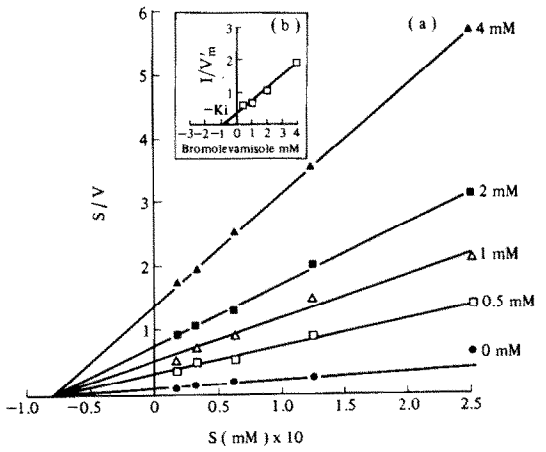

Fig. 3. Inhibition of placenta diamine oxidase by bromo-levamisole at pH 7.4. (A) Hanes plots of substrate concentration (S) divided by initial velocity (V) versus substrate concentration (S) at different concentrations of bromo-levamisole. (B) Determination of K_i. (The error bars are smaller than the symbols used to represent the experimental points.)

competitive inhibitors such as phosphate, orthovanadate or phenylphosphonic acid [29]. Therefore, it is more useful to study the pharmacological activity–inhibition relationship with uncompetitive inhibitors than with competitive inhibitors which have many biological targets in the cell.

Inhibition of DAO

Putrescine (1,4-diaminobutane) and histamine (2-(4-imidazolyl) ethylamine) are considered as natural potential substrates for DAO. In the present study putrescine is used as a substrate and Table 1 records the I_{50} obtained. The inhibitory potency of tested compounds is:

$$L \gg M > A, B > C > I > D > J > K > H, F, G.$$

Compound L, tested before [30], is the most potent inhibitor. We show that cimetidine (compound M) is also a potent inhibitor. Since imidazole (compound J) and guanidine (data not shown) are inhibitors, this explains that substituted furan or ethenyl diamine of compound O are not favourable. The I_{50} obtained with compounds A, B and I suggests that the substitution of the thiazole ring on C_2 is responsible for inhibition. Considering the similar inhibition observed for compounds A and B, the phenyl ring is not necessary, but it increases the inhibition of compound C compared to J.

Only bromo-levamisole was studied with respect to the nature of inhibition and we obtained a non-competitive type (Fig. 3), while a competitive one has already been reported for DAO from human placenta [2]. However, these authors found a non-competitive type for levamisole.

Pharmacology and ALP-DAO inhibition relationship

It is known that several compounds inhibit ALP or DAO and possess an immunomodulating effect *in vivo* and/or *in vitro*. These properties are a feature of levamisole but also compound L [10], N [31] and J [32]. We show that cimetidine, which interacts with the H_2-type receptors bearing T suppressor lymphocytes [33], inhibits ALP and DAO. Furthermore, ranitidine, another H_2-antagonist, does not inhibit ALP and DAO and has no immunomodulating effect.

It is difficult to correlate an enzyme inhibition *in vitro* with a biological test using a cell. However, with these preliminary results, it is reasonable to assume that inhibition study of ALP and DAO may help to select immunomodulating compounds. We suggest that the uncompetitive inhibition of ALP is very important to select these compounds as is shown for cimetidine. Our data using human hepatic ALP and human placenta DAO is considered similar to that which is obtained using enzymes from leukocyte origin. The human leukocyte ALP is inhibited by levamisole [34, 35] and derives from the same genetic loci as the human hepatic ALP [36]. The DAO from leukocyte origin is considered as having the same features as the enzyme from placenta origin [10]. Therefore, the involvement of ALP or DAO in the immunological process is only possible if the concentrations of compounds which inhibit the enzymes have also an immunomodulating effect. This takes place with compounds L, M and N previously tested *in vitro*.

Earlier reports [31, 37, 38] show that the increase in the function of suppressor T cell by compound N might be due to the inhibition of AMP-phosphodiesterase (PDE) or an antagonist effect on adenosine receptors. As the phosphoprotein-phosphatase of ALP is presently confirmed, the inhibition of this latter activity may interfere with the inhibition of PDE. The compound N increases cAMP concentration which activates cAMP-dependent kinases and inhibits dephosphorylation of phosphoproteins by these same kinases. Therefore the increase of phosphoproteins in suppressor T cells may regulate the metabolic activities of these cells.

CONCLUSION

In the present study, we show that the structure–inhibition relationship of ALP and DAO allows the selection of cimetidine, an immunomodulating compound, as inhibitor of these two enzymes. This drug presents an imidazole ring which is necessary to inhibit ALP and DAO. The methylene chain is more favourable to ALP and the guanidine group more favourable to DAO. Nevertheless, further investigations should be performed to find other compounds such as cimetidine. If such molecules exist, it should be possible to synthesize a drug towards the active site of ALP and DAO with potential immunomodulating activities. Furthermore the way in which the compounds act can be investigated with this method, because ALP and DAO are respectively involved in protein phosphorylations and the metabolism of polyamines, two events which are correlated with cellular regulations.

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