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# Basic models for differential inhibition of enzymes

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ABSTRACT

The possible preferential action exerted by an inhibitor on the transformation of one of two agonist substrates catalyzed by the same enzyme has recently been reported in studies on aldose reductase inhibition. This event was defined as "intra-site differential inhibition" and the molecules able to exert this action as "differential inhibitors". This work presents some basic kinetic models describing differential inhibition. Using a simple analytic approach, the results show that differential inhibition can occur through either competitive or mixed type inhibition in which the inhibitor prevalently targets the free enzyme. The results may help in selecting molecules whose differential inhibitory action could be advantageous in controlling the activity of enzymes acting on more than one substrate.



## 1. Introduction

The susceptibility to allosteric inhibition enables enzymes to respond to cell conditions in order to meet metabolic needs. Furthermore enzyme inhibition is one of the most common strategies used to control specific enzymes or metabolic pathways. Different models of enzyme inhibition, used to explain experimental inhibition data, focus on the relative ability of the inhibitor to interact with the free enzyme and/or with the enzyme-substrate complex. This approach leads to a pragmatic definition of the active and inhibition sites, and the related influence between them is considered as the rationale underlying the inhibitor's behavior.

A significant aspect of enzyme inhibition studies concerns the ability of several enzymes to act on different competitive alternative substrates. The first issue is to discriminate between the real ability of the enzyme to act on different substrates and the presence in the enzyme preparations of different enzyme molecules (i.e. isoenzymatic forms or modified forms). To solve this problem a methodological approach was proposed to analyze systems in which two substrates were simultaneously present, which provides insight on whether the transformations could be ascribed to one or to two different catalytic sites [1]. The analysis of a system consisting of one enzyme with two competing substrates using the steady-state kinetic approach leads to a kinetic equation which reveals an apparent mutual inhibitory effect of the two substrates undergoing transformation [2–5]. This analysis thus evaluates the real catalytic effectiveness of an enzyme on substrates simultaneously present in the biological systems. The effect of substrates competing for the same enzyme on metabolic pathways has been recently considered [6]. In addition the use of competing substrates has been proposed to determine the steady state kinetic parameters for the agonist substrate [7]. Similarly, other authors have used alternative substrates to probe multi-substrate enzyme mechanisms [3,8].

We recently proposed a new strategy regarding the inhibition of enzymes that are able to act on different substrates, in which the inhibitor selectively intervenes on the enzyme activity depending on the substrate the enzyme is working on. Such an intra-site specific inhibitory action was termed as "differential inhibition" and the effectors able to exert this action as "differential inhibitors" (DIs) [9]. The usefulness of this approach was highlighted in studies on aldose reductase (AR), an enzyme targeted for decades with specific and highly powerful inhibitors (ARIs) in order to find drugs for the prevention of diabetes complications (for a review see [10]). The ability of the enzyme to reduce both hydrophilic substrates, such as aldoses (thus giving rise to cell damage) and hydrophobic aldehydes, such as HNE (thus removing a cytotoxic product of lipid peroxidation), raised doubt as to the effective gain in inhibiting the enzyme. This doubt is supported by a general failure in developing effective drugs from ARIs. Indeed, we also proved the potential of AR to undergo differential inhibition [9], and a new generation of enzyme inhibitors, aldose reductase differential inhibitors (ARDIs), is under investigation.

The first step in revealing DIs for enzymes with respect to two different substrates involves evaluating the inhibitory effectiveness

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of the inhibitor in terms of the two different substrates assayed separately. However the ability of the inhibitor to act as a DI must be more realistically verified by testing the inhibitory action when both substrates are present in the assay.

This paper presents the simple kinetic analysis of a system in which the catalytic activity of an enzyme on two agonist substrates is differently targeted by a DI.

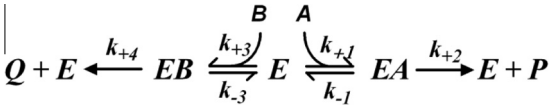
## 2. Methods

The present analysis assumes that classical Michaelis–Menten kinetics is obeyed, that agonist substrates are mutually exclusive and that the inhibitor combines reversibly with the enzyme and or with the enzyme substrate complexes. Rate equations at zero time, derived for different models of differential inhibitions, are graphically represented essentially by double reciprocal plots [11].

## 3. Kinetic models and discussion

### 3.1. Two agonist substrates for the same enzyme

The following scheme describes a reaction model in which two different substrates (*A* and *B*) are recognized and transformed into the corresponding products (*P* and *Q*, respectively) by the same enzyme (*E*) complying with a simple kinetic model:




(Scheme 1)

The steady state analysis of the enzymatic reactions [5] leads to the final kinetic equations (Eqs. (1) and (2)) for the transformation of the two substrates.

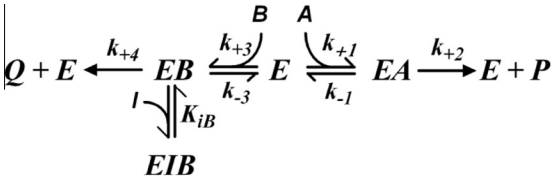
$$-\frac{d[A]}{dt} = v_p = \frac{V_A[A]}{K_A\left(1+\frac{[B]}{K_B}\right)+[A]} \tag{1}$$

$$-\frac{d[B]}{dt} = v_q = \frac{V_B[B]}{K_B(1+\frac{[A]}{K_A})+[B]} \tag{2}$$

In these equations $V_A = k_{+2}E_T$, $K_A = \frac{k_{-1}+k_{+2}}{k_{+1}}$, $V_B = k_{+4}E_T$ and $K_B = \frac{k_{-3}+k_{+4}}{k_{+3}}$. The terms $V_A$, $V_B$ and $K_A, K_B$ represent the maximum reaction rates and the Michaelis–Menten constants for the transformation of *A* and *B*, respectively, measured in the presence of the total enzyme concentration ($E_T$) in the absence of the agonist substrate. These equations highlight that when the two agonist substrates are simultaneously present, they reciprocally act as competitive inhibitors. In this model a differential inhibitor is defined as a molecule with the ability to specifically act only on the transformation of one of the two substrates. Kinetic models for ideal differential inhibitors were devised.

### 3.2. Uncompetitive differential inhibition

The simplest model describing the targeting of an inhibitor exclusively on one of the two competing substrates is reported in the following scheme, in which the action of the inhibitor (*I*) is limited to the binding to the complex *EB*, thus depicting for substrate *B* a model of an uncompetitive type of inhibition:




(Scheme 2)

This model admits that the structural differences between the two substrates will determine, once bound on the enzyme, the exposure of a different interacting surface for the inhibitor. Thus, only the *EB* complex is targeted by the inhibitor. The general kinetic equations for *A* and *B* transformation are:

$$v_p = \frac{d[P]}{dt} = k_{+2}[EA]$$

$$v_q = \frac{d[Q]}{dt} = k_{+4}[EB]$$

By applying the steady state condition for *EA* and *EB* and considering the complex *EIB* at the equilibrium, the following equations can be written:

$$k_{+1}[E][A] = (k_{-1}+k_{+2})[EA]$$

$$k_{+3}[E][B] = (k_{-3}+k_{+4})[EB]$$

$$K_{iB} = \frac{[EB][I]}{[EIB]}$$

Taking into account the mass balance for the enzyme:

$$E_T = [E]+[EA]+[EB]+[EIB]$$

a kinetic equation can be formulated (see Supplementary Material, Appendix I) for the transformation of both *A* and *B* (Eqs. (3) and (4), respectively)

$$v_p = \frac{k_{+2}E_T[A]}{K_A\left[1+\frac{[B]}{K_B}\left(1+\frac{[I]}{K_{iB}}\right)\right]+[A]} \tag{3}$$

$$v_q = \frac{k_{+4}E_T[B]/\left(1+\frac{[I]}{K_{iB}}\right)}{K_B\left(1+\frac{[A]}{K_A}\right)/\left(1+\frac{[I]}{K_{iB}}\right)+[B]} \tag{4}$$

As can be seen from Eq. (4), the transformation of substrate *B* in the presence of the agonist substrate *A* and the differential inhibitor *I* is affected both in terms of $V_{max}$ as a result of the inhibitor action and in terms of apparent $K_M$ as a result of the combined action of both the inhibitor and the agonist substrate (Fig. 1). In this case, changes in $K_B^{app}$ values are linked to the relative values of the inhibitory impact factor for both the inhibitor $\left(1+\frac{[I]}{K_{iB}}\right)$ and the agonist substrate $\left(1+\frac{[A]}{K_A}\right)$. As also it emerges from Eq. (3), the inhibition of the transformation of *B* negatively affects the transformation of substrate *A*, although the latter was not directly targeted by the inhibitor. In fact the generation of the ternary complex *EIB*, by reducing the availability of the enzyme, leads to a general decrease in $v_p$. More specifically, at any finite value of both [*B*] and [*I*], $K_A^{app}$ increases with the increase of both [*I*] and [*B*], while $V_{max}$ remains constant (Fig. 2).

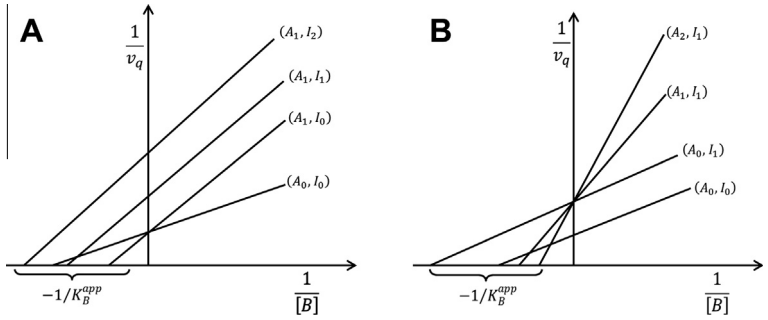



**Fig. 1.** Double reciprocal plots of substrate *B* transformation for differential uncompetitive inhibition. *Panels a* and *b* show the effect on substrate *B* transformation exerted by the inhibitor and substrate *A*, respectively, as predictable from Eq. (4). In both panels the relative concentration values of substrate *A* ($A_0$, $A_1$, $A_2$, with $A_1 < A_2$ and $A_0 = 0$) and inhibitor ($I_0$, $I_1$, $I_2$, with $I_1 < I_2$ and $I_0 = 0$) are indicated on each curve. The effect exerted on substrate *B* transformation by both substrate *A* and the inhibitor present alone in the reaction mixture (curve $A_1$, $I_0$, *Panel a* and curve $A_0$, $I_1$, *Panel b*, respectively) is also reported.

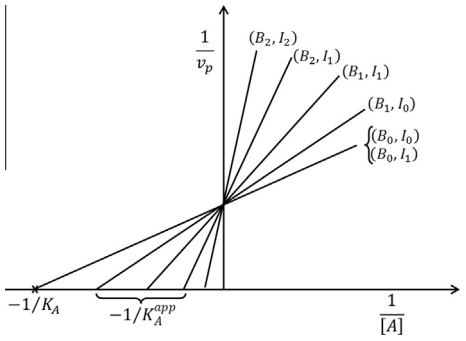
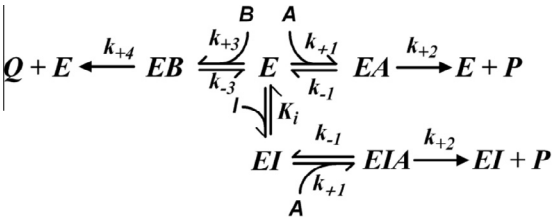



**Fig. 2.** Double reciprocal plot of substrate *A* transformation for differential uncompetitive inhibition. Referring to Scheme 2, the effect on substrate *A* transformation exerted by the inhibitor and substrate *B*, as predictable from Eq. (3) is reported. The relative concentration values of substrate *B* ($B_0$, $B_1$, $B_2$, with $B_1 < B_2$ and $B_0 = 0$) and inhibitor ($I_0$, $I_1$, $I_2$, with $I_1 < I_2$ and $I_0 = 0$) are indicated on each curve. The effect exerted on substrate *A* transformation by both substrate *B* and the inhibitor present alone in the reaction mixture (curve $B_1$, $I_0$, and curve $B_0$, $I_1$, respectively) is also reported.

### 3.3. Competitive differential inhibition

The following scheme refers to the action of an inhibitor *I* which binds to the free enzyme competing only with the binding of substrate *B*; at the same time substrate *A* may still bind the enzyme and undergo transformation, irrespectively of the presence of the inhibitor.




(Scheme 3)

This model represents the case in which the structural differences between the two substrates and the features of the enzyme active site lead to a different interaction pattern in the generation of the enzyme-substrate complexes. The transformation rate for both *A* and *B* can be expressed as:

$$v_p = \frac{d[P]}{dt} = k_{+2}([EA] + [EIA])$$

$$v_q = \frac{d[Q]}{dt} = k_{+4}[EB]$$

By applying the steady state condition for *EA*, *EB* and *EIA* and considering the complex *EI* at the equilibrium, the following equations can be written:

$$k_{+1}[E][A] = (k_{-1} + k_{+2})[EA]$$

$$k_{+1}[EI][A] = (k_{-1} + k_{+2})[EIA]$$

$$k_{+3}[E][B] = (k_{-3} + k_{+4})[EB]$$

$$K_i = \frac{[E][I]}{[EI]}$$

Taking into account the mass balance for the enzyme:

$$E_T = [E] + [EI] + [EA] + [EIA] + [EB]$$

a kinetic equation can be formulated (see Supplementary Material, Appendix IIa) for the transformation of both *A* and *B* (Eqs. (5) and (6), respectively)

$$v_p = \frac{k_{+2}E_T[A]}{K_A\left(1 + \frac{\frac{[B]}{K_B}}{1 + \frac{[I]}{K_i}}\right) + [A]} \tag{5}$$

$$v_q = \frac{k_{+4}E_T[B]}{K_B\left(1 + \frac{[A]}{K_A}\right)\left(1 + \frac{[I]}{K_i}\right) + [B]} \tag{6}$$

The above equations highlight that although the DI inhibits the transformation of substrate *B* (Eq. (6), Fig. 3, *Panel a*), with the increase in its concentration it leads to a progressive enhancement of $v_p$ (Eq. (5)). This is clearly evident from the progressive increase in the apparent affinity ($K_A^{app}$ decreases) with the increase in inhibitor concentration (Fig. 3, *Panel b*) Indeed, the secondary plot of $K_A^{app}$ versus the reciprocal of the inhibitory impact factor $(1 + \frac{[I]}{K_i})$ (Fig. 3, *Panel c*) shows the effect of *I* on *A* transformation. This effect only depends on the removal of the inhibition exerted by the agonist substrate. This will imply that the apparent effectiveness of *I* is linked to *B* concentration, being clearly null when only *A* is present (i.e. $K_A^{app} = K_A$ at any *I* concentration).

In conclusion, in the case of competitive differential inhibition, there is not only a decrease in *Q* formation, due to the DI, but also to an increase in *P* formation because of the relief of the competition of *B* with respect to *A*.

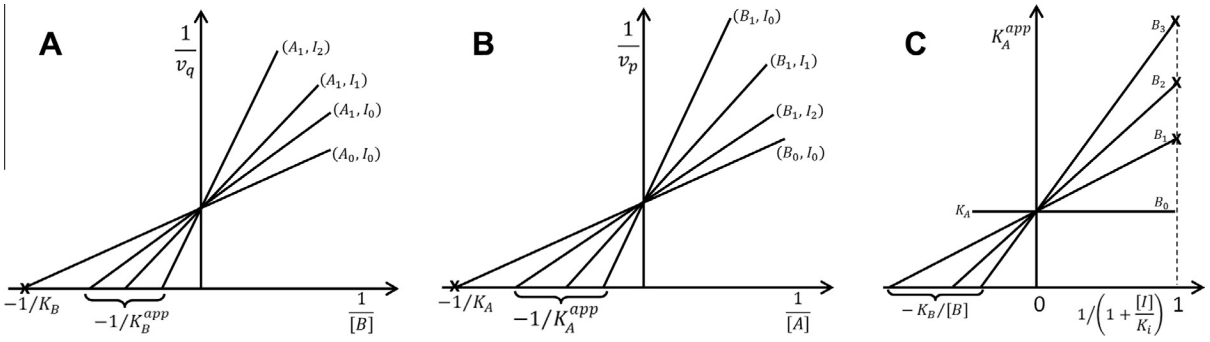
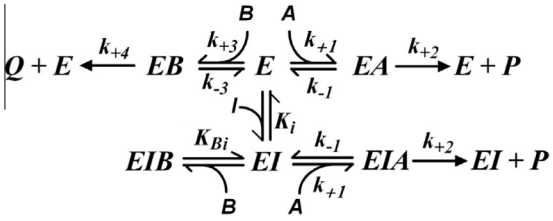



**Fig. 3.** Graphical representation of differential competitive inhibition. *Panels a* and *b* show the effect exerted by the inhibitor on the transformation of substrates *B* and *A*, respectively, as predictable from Eqs. (6) and (5) analyzed by double reciprocal plot. In both panels the relative concentration values of inhibitor ($I_0$, $I_1$, $I_2$, with $I_1 < I_2$ and $I_0 = 0$) and substrates *A* ($A_0$, $A_1$, with $A_0 = 0$) (*Panel a*) or *B* ($B_0$, $B_1$, with $B_0 = 0$) (*Panel b*) are indicated on each curve. The effect exerted on substrate *B* transformation by both substrate *A* and the inhibitor present alone in the reaction mixture (curve $A_1$, $I_0$, *Panel a* and curve $A_0$, $I_1$, *Panel b*, respectively) are also reported. *Panel c* shows the effect of the inhibitor concentration expressed as $1/\left(1+\frac{[I]}{K_i}\right)$ on $K_A^{app}$ at different concentrations of substrate *B* ($B_0$, $B_1$, $B_2$, with $B_1 < B_2$ and $B_0 = 0$). The abscissa values of 0 and 1 refer to inhibitor concentrations of $\infty$ and 0, respectively.

### 3.4. Mixed type differential inhibition

The kinetic model for mixed type differential inhibition is reported in the following scheme:




(Scheme 4)

It differs from the differential competitive inhibition due to the possibility of generating the ternary complex *EIB* which, in turn, does not evolve to products. At the same time substrate *A* may still bind the enzyme and undergo transformation, irrespectively of the presence of the inhibitor.

In this case, in addition to the equations defining the complete competitive differential inhibition (see Supplementary Material, Appendix IIa) $K_{Bi}$, the dissociation constant of the ternary complex *EIB*, must be considered:

$$K_{Bi} = \frac{[EI][B]}{[EIB]}$$

Again, by assuming the steady state conditions for *EA*, *EB* and *EIA*, and assuming the complex *EIB* at the equilibrium, a kinetic equation can be formulated (see Supplementary Material, Appendix III) for the transformation of both *A* and *B* (Eqs. (7) and (8), respectively):

$$v_p = \frac{k_{+2}E_T[A]}{K_A\left(1+\frac{[B]\left(1+\frac{[I]K_B}{K_iK_{Bi}}\right)}{K_B\left(1+\frac{[I]}{K_i}\right)}\right)+[A]} \tag{7}$$

$$v_q = \frac{k_{+4}E_T[B]}{K_B\left(1+\frac{[I]}{K_i}\right)\left(1+\frac{[A]}{K_A}\right)+[B]\left(1+\frac{K_B[I]}{K_iK_{Bi}}\right)} \tag{8}$$

Regarding the uncompetitive type of inhibition, also in this case the generation of the ternary complex can also affect the transformation of substrate *A*, which the model imposes should not be directly influenced by the inhibitor.

Although, depending on the mechanism of action, the transformation of *A* can be affected by the differential inhibitors (i.e. uncompetitive and mixed types of inhibition), the above described inhibitors, which directly intervene exclusively on *B* transformation, can be considered as "*complete*" DIs. A complete competitive inhibitor will be able to prevent the reaction of its target substrate since it has absolutely no effect on the agonist. However, there may be a "*partial*" competitive DI, able to also affect the *A* transformation (although to a lesser extent than *B*).

Still looking at Fig. 2, we may consider the case when *A* transformation can be affected both in terms of the ability of *A* to interact with the *EI* complex or/and the ability of the *EIA* complex to evolve to *P*. In this case, given that $k_{+5}$ and $k_{-5}$ are the kinetic constants for the formation and dissociation of the *EIA* complex, respectively, and $k_{+6}$(with $k_{+6} \leqslant k_{+2}$)is the kinetic constant of the dissociation of the ternary complex *EIA* to *P*, equations for $v_p$ and $v_q$ as a function of substrates and inhibitor concentrations (Eqs. (9) and (10)) can be derived (see Supplementary Material, Appendix IIb):

$$v_p = \frac{\left(\frac{k_{+2}}{1+\frac{\alpha[I]}{K_i}}+\frac{k_{+6}}{1+\frac{K_i}{\alpha[I]}}\right)E_T[A]}{K_A\left(\frac{1+\frac{[I]}{K_i}+\frac{[B]}{K_B}}{1+\frac{\alpha[I]}{K_i}}\right)[A]} \tag{9}$$

$$v_q = \frac{k_{+4}E_T[B]}{K_B\left[\left(1+\frac{[I]}{K_i}\right)+\frac{[A]}{K_A}\left(1+\frac{\alpha[I]}{K_i}\right)\right]+[B]} \tag{10}$$

in which

$$\alpha = \frac{K_A}{K'_A} \text{ with } K'_A = \frac{k_{-5}+k_{+6}}{k_{+5}}$$

The term $\alpha$ represents a relative affinity factor for the binding of substrate *A* between the free enzyme *E* and the enzyme-inhibitor complex *EI*. It is evident that a progressive increase of $\alpha$ will favor the flux of substrate *A* through the ternary complex formation with a decrease in the apparent affinity constant $K_A^{app}$ and, where the $k_{+2} > k_{+6}$,with a decrease in $k_{cat}$. Clearly, when $k_{+6} = k_{+2}$, an increase of $\alpha$ will only relieve the competitive inhibition exerted on *A* transformation by the agonist substrate *B* (see Eq. (5)).

On the basis of these considerations, in order to maximize the differential inhibitory effect, the search for DIs should address the molecules able to exert a competitive mode of action.

In the case of a mixed type of DIs, the preferential targeting of the free enzyme with respect to the *EB* complex, should maximize the effectiveness of the differential inhibition.

Finally, the relative affinity of the enzyme for the two substrates is another factor which could impact on the effectiveness of the differential inhibition. In fact, in both the competitive and mixed type differential inhibition, a progressive enhancement of the *apparent* $K_B$ constant takes place with the increase in the $K_B/K_A$ ratio (Eqs. (6) and (8)). In the same conditions there is a parallel loss of sensitivity of $K_A$ to the competition effect of the agonist substrate *B* at different inhibitor concentrations (Eqs. (5) and (7)). This condition could be favorable in the search for DIs. This situation occurs in the case of AR, the enzyme which stimulated the present analysis and which has been proved to be susceptible to differential inhibition. In fact AR displays marked differences between the kinetic parameters for the two relevant substrates (i.e. glucose and HNE), whose differential inhibition may be beneficial for the cell. The significantly higher $K_M$ values reported for glucose (from approximately 35 to 200 mM) [12–17] with respect to HNE (approximately 35 μM) [9,18,19] may lead to the possibility of identifying ARDIs which could be successfully developed as effective drugs in diabetic complications.

The definition of basic models of differential inhibition clearly shows the most useful direction to look and search for differential inhibitors. The most relevant feature for DIs should be their ability to interact with the free enzyme rather than with the enzyme substrate complexes. This competitive behavior could also help in displaying a differential inhibition for molecules acting through a mixed type inhibition model. Uncompetitive inhibition is a rare event in the metabolic enzyme control [20]. Nevertheless, uncompetitive inhibitors are often proposed for enzyme targeting [21–24]. Uncompetitive inhibitors would not be potential DIs because of the unavoidable indirect effect on the transformation of the agonist substrate. We believe that the search for DIs will require a completely new approach which by necessity must take into account the different patterns of interaction of the enzyme with the two agonist substrates. A new design in experimental inhibition measurements and a different computational analysis will thus be required. It is not enough simply to assess the inhibitory potency, which so far has been the only factor considered when selecting the inhibitors.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.030.

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