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Inhibition of rabbit lung angiotensin converting enzyme by idrapril

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Abstract—Idrapril, the prototype of a new class of angiotensin converting enzyme (ACE) inhibitors, competitively inhibited, with nanomolar apparent K_i, the hydrolysis of hippuryl-glycyl-glycine by rabbit lung ACE. The pre-steady-state analysis of this tight-binding inhibition showed it to be characterized by slow kinetics, but at variance with what was found for enalaprilat in the same conditions, idrapril appeared to act through a simple, single step mechanism. Kinetic K_i and k_{on} and k_{off} values were 470 pM, $3.0 \pm 1.5 \times 10^6\ M^{-1}\ sec^{-1}$ and $1.4 \pm 0.3 \times 10^{-3}\ sec^{-1}$, respectively.

Idrapril ((+)-(1*S*,2*R*)-2-{[*N*-(2-hydroxyamino-2-oxoethyl)-*N*-methylamino]carbonyl}cyclohexane-1-carboxylic acid), the prototype of a new class of angiotensin converting enzyme (ACE*) inhibitors, is characterized by a hydroxamic group as ligand of the zinc ion present at the catalytic site of ACE and by the substitution of the proline-like moiety, a common feature in many of the known ACE inhibitors, with an alicyclic dicarboxylic acid [1, 2]. The active moieties of clinically effective ACE inhibitor drugs, e.g. captopril [3], enalaprilat [3], ramiprilat [4] and lisinopril [5], are known to fulfil the criteria reported by Morrison [6] for being considered slow- and tight-binding inhibitors. Consequently the kinetics of their interaction with the enzyme cannot be described by classical kinetic analysis, but requires the use of the equations developed by Williams and Morrison [7] and Cha [8] for these kinds of inhibitors.

It has been recently reported that pulmonary ACE possess two inhibitory binding sites that have been shown to be catalytically active, even if with a distinct substrate specificity [9], but the role of the second site in the interaction with typical ACE substrates and inhibitors is still under study.

The aim of this study was to define the mechanism of the inhibition of rabbit lung ACE by idrapril, in comparison with enalaprilat, using hippuryl-L-glycyl-L-glycine (Hip-Gly-Gly) as a substrate and taking into account the possible role of the two active sites of ACE.

Materials and Methods

ACE purified from rabbit lung membrane (A6778) was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The synthetic substrate Hip-Gly-Gly (Peninsula Laboratories Inc., CA, U.S.A.) was dissolved in 50 mM Hepes buffer, pH 8, containing 400 mM Na_2SO_4 and 300 mM NaCl (unless otherwise stated in the text). The purified enzyme was dissolved in the same buffer (final concentrations ranged from 0.4 to 4.4 nM). Stock solutions (1 mM) of the inhibitors were prepared in distilled water with the aid of stoichiometric quantities of sodium bicarbonate. Assays were conducted at 37° and initiated by addition of ACE to the mixture of substrate and inhibitor, unless otherwise stated in the text. Typical volumes were: substrate 500 μL, inhibitor 70 μL and enzyme 130 μL. At different times (see figures), 70 μL of incubation mixture were sampled and added to 50 μL of 0.5 N perchloric acid in order to stop the reaction. Cleaved hippuric acid was extracted and quantitated by HPLC, according to Neels *et al.* [10].

All kinetic calculations were performed by computerized linear or non-linear regression analysis (Enzfitter program for PC, Elsevier Science Publishers BV, Amsterdam, NL). K_m and k_{cat} values were derived from Michaelis–Menten plots. Methods for analysis of the pre-steady-state were adapted from Williams and Morrison [7] and Cha [8]. No more than 5% of the substrate was hydrolysed during the assay, the ratio of inhibitor (I) concentration to enzyme (E) concentration was at least 6 and that of substrate to enzyme was about 10^7. Under these conditions K_{obs}, a pseudo-first-order rate constant for the inhibitor-induced decrease in the reaction velocity, can be calculated [7] from the integrated equation:

$$P = V_s \cdot t - (V_s - V_o)(1 - e^{-K_{obs} \cdot t})/K_{obs} \qquad (1)$$

By fitting data for product formation (P) as a function of time (t) to this equation, it was possible to determine V_s (steady-state velocity) and V_o (zero-time velocity) at the same time as K_{obs}.

Results

Enzyme concentration was preliminarily calculated from the activity measured with angiotensin I and hippuryl-L-histidyl-L-leucine as substrates, for which k_{cat} values of 600 and 15,600 min^{-1} had been reported [11]. Similar values for the concentration of the stock ACE solution (34 and 31 nM, respectively) were obtained in these two cases.

In order to verify whether Hip-Gly-Gly is hydrolysed by only one or both the active sites of ACE, the residual Hip-Gly-Gly hydrolysing activity after addition of increasing lisinopril concentrations was measured. Lisinopril was used because it had been shown to bind to both the domains of ACE [9]. From the value of the x-intercept in Fig. 1, not significantly different from the calculated value for ACE concentration, it appears that 1 mol of inhibitor per mol of enzyme was sufficient to inhibit Hip-Gly-Gly hydrolysis by about 90%.

The interaction of idrapril with ACE was initially examined by classical steady-state kinetics. Michaelis–Menten parameters for the hydrolysis of Hip-Gly-Gly in the presence of 300 mM NaCl were: $K_m = 9.3 \pm 1.5$ mM and $k_{cat} = 120{,}000 \pm 20{,}000\ min^{-1}$ (mean values ± SE, N = 6). In the presence of 6, 12 and 25 nM idrapril, apparent K_m (K'_m) for 0.4 nM ACE increased to 58 ± 6, 105 ± 6 and 221 ± 17 nM, respectively, whereas virtually constant k_{cat} values ($150{,}000 \pm 8000$, $150{,}000 \pm 5250$ and $170{,}000 \pm 9860\ min^{-1}$, means ± approximated SE evaluated by the regression program on seven experimental points) were observed. Idrapril thus behaved as a competitive inhibitor. K_i value, obtained from the expression $K_i = [I]/(K'_m/K_m - 1)$ [12], was 1.13 ± 0.01 nM (mean ± SE, N = 3), suggestive of idrapril being a tight-binding inhibitor of ACE. In separate experiments, IC_{50} values were shown to depend on the concentration of enzyme: 3.7 ± 0.1, 8.0 ± 0.4 and 14.5 ± 0.1 nM (means ± approximated SE evaluated by the regression program on nine experimental points) for 0.4, 0.75 and 4.4 nM ACE, respectively. This confirmed that idrapril is a tight-binding inhibitor [7] and a more appropriate kinetic

* Abbreviations: ACE, angiotensin converting enzyme; Hip-Gly-Gly, hippuryl-L-glycyl-L-glycine.

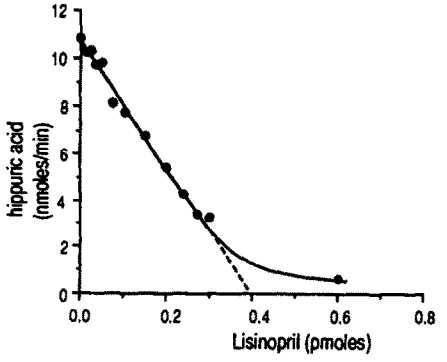

Fig. 1. Titration of active sites in ACE by binding of the competitive inhibitor lisinopril. Purified pulmonary ACE (0.34 pmol) was incubated with 0.0152–0.6 pmol of lisinopril ($K_i \leq 90$ pM) at room temperature for 30 min in 50 mM Hepes, pH 8, containing 0.3 M NaCl and 0.4 M Na_2SO_4, in a volume of 20 μL. Residual enzyme activity was then determined by addition of 50 μL of Hip-Gly-Gly (6 mM) dissolved in the same buffer and measuring initial rates after 6 min of incubation at 37° (substrate consumption was <5%). Intersection of the extrapolated linear portion of the curve (broken line) with the x-axis gives the number of pmoles of bound lisinopril: 0.39.

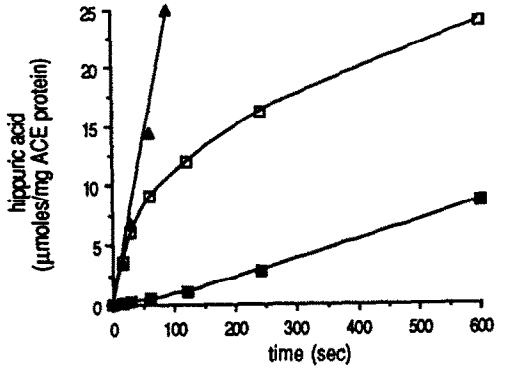

Fig. 2. Progress curves for inhibition of rabbit lung ACE by 50 nM idrapril. (□) Reaction started by the addition of enzyme; (■) reaction started by the addition of substrate after a 4 hr incubation of the enzyme and the inhibitor at room temperature; (▲) control curve with no inhibitor added. Assays were performed at 37° in 50 mM Hepes, pH 8, containing 300 mM NaCl, 400 mM Na_2SO_4. Final concentrations of ACE and Hip-Gly-Gly were 3.7 nM and 30 mM, respectively. At the established times aliquots of the mixtures were added to 50 μL of 0.5 N $HClO_4$ to stop the reaction. Formed hippuric acid was quantitated by HPLC. Each point is the mean of duplicate determinations.

analysis was undertaken. Time course of the enzymatic reaction was examined with and without preincubation of ACE with 50 nM idrapril. As shown in Fig. 2, in the absence of inhibitor maximal rate was achieved almost instantaneously and remained constant in the considered time range. In the presence of idrapril, when the reaction was started by adding the enzyme to a mixture of inhibitor and substrate, the high initial velocity decreased to a lower steady-state value and a downward concave curve was obtained. On the other hand, when the enzyme was preincubated with idrapril for 4 hr at room temperature before starting the reaction with substrate, an upward concave curve was obtained tending to the same steady-state rate as above. This behaviour clearly indicated that the equilibrium between ACE and idrapril is not rapidly established, i.e. idrapril is a slow-binding inhibitor of ACE. Progress curves characterizing the slow development of ACE inhibition by idrapril and enalaprilat (a well known tight- and slow-binding ACE inhibitor), at different substrate or inhibitor concentrations, are reported in Fig. 3. In every case, the experimental points were well fitted by curves described by Eqn 1. Calculated parameters K_{obs}, V_s and V_o were then used to compute the rate constant for the dissociation reaction of the final enzyme–inhibitor complex, $k_{off} = K_{obs} \cdot V_s/V_o$, according to Morrison [6].

The diagnostic plot of $1/(K_{obs} - k_{off})$ versus substrate concentration, reported in Fig. 4, provided information on the inhibition mechanism. The straight lines with positive slope obtained for both tested compounds indicated again competitive inhibition [8]. Slow competitive inhibition is consistent with at least two kinetic mechanisms [3, 6], which are:

Mechanism A:

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} EI.$$

In this case a low value for k_{on} accounts for the reduced velocity of the EI complex formation, $K_i = k_{off}/k_{on}$ and

$$k_{obs} = k_{off} + k_{on}[I]/(1 + [S]/K_m). \quad (2)$$

Or mechanism B:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftarrows}} EI \underset{k_4}{\overset{k_3}{\rightleftarrows}} EI^*.$$

In this case $K_i = k_2/k_1$, but the rate-limiting step is the conversion of the complex EI to a more stable one EI*. The overall reaction ($E + I \rightleftarrows EI^*$) is characterized by $K_i^* = K_i \cdot k_4/(k_3 + k_4)$, with $k_4 = k_{off}$, and

$$K_{obs} = k_4 + k_3[I]/\{[I] + K_i(1 + [S]/K_m)\}. \quad (3)$$

The two mechanisms can be distinguished by the different effects of substrate and inhibitor concentration on K_{obs}, as described by Eqns 2 and 3. The analysis of the substrate-dependence may be done by plotting data as reported in Fig. 4. In fact, the intercept on the horizontal axis ([S]) would be equal to $-K_m$ with mechanism A and to $-K_m([I] + K_i)/K_i$ for mechanism B. With idrapril, the intercept was 8 mM, close to the calculated K_m (9.3 mM); with enalaprilat the intercept was 60 mM, clearly different from K_m. These results suggested that idrapril follows mechanism A, whereas enalaprilat follows mechanism B. The applicability of Eqn 3 was then verified by plotting the dependence of $1/(K_{obs} - k_{off})$ on $1/[I]$, as shown in Fig. 5A. Mechanism B may be an appropriate description of the inhibition by enalaprilat, as the regression line had a positive y-intercept equal to 10 ± 5 sec (mean ± approximated SE evaluated by the regression program on four experimental points). From y- and x-intercepts [$1/k_3$ and $-1/K_i(1 + [S]/K_m)$, respectively] values for k_3 (0.1 sec^{-1}), K_i (22 nM) and K_i^* (60 pM) were calculated. For idrapril the line passed through the origin (y-intercept = 3 ± 8 sec, N = 6), and it was not possible to determine the kinetic parameters according to mechanism B. Similar results were obtained for idrapril when the analysis of the pre-steady-state was carried out in the presence of a non-saturating concentration of NaCl (2 mM, K_m = 20 mM and k_{cat} = 34,000 min^{-1}), in order to establish how the kinetic parameters were affected by the chloride concentration. The $1/(K_{obs} - k_{off})$ on $1/[I]$ plot was linear with y-intercept = -2 ± 5 sec, so that also in these conditions it was not possible to apply Eqn 3. On the other hand, the linear dependence of K_{obs} on inhibitor concentration, illustrated in Fig. 5B, indicated that Eqn 2

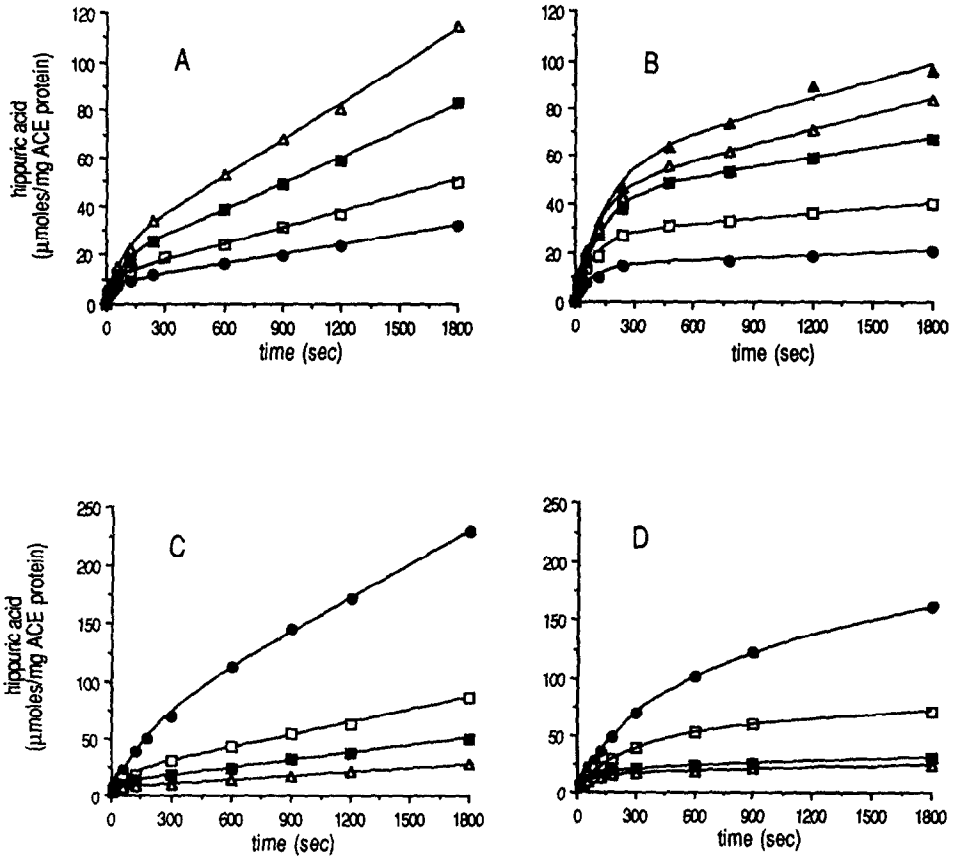

Fig. 3. Representative tracings of kinetic curves for the slow onset of inhibition at different concentrations of inhibitors or substrate. (A) Idrapril 50 nM or (B) enalaprilat 10 nM and Hip-Gly-Gly: (●) 15 mM, (□) 30 mM, (■) 45 mM, (△) 60 mM, (▲) 75 mM; (C) Hip-Gly-Gly 30 mM and idrapril: (●) 10 nM, (□) 25 nM, (■) 50 nM, (△) 100 nM; (D) Hip-Gly-Gly 30 mM and enalaprilat: (●) 2.5 nM, (□) 5 nM, (■) 10 nM, (△) 20 nM. Assays were performed at 37° in 50 mM Hepes, pH 8, containing 300 mM NaCl, 400 mM Na_2SO_4, and initiated by addition of ACE to mixtures of substrate and inhibitor ([I]/[E] was >6). At the established times aliquots of the mixtures were added to 50 μL of 0.5 N $HClO_4$ to stop the reaction. Formed hippuric acid was quantitated by HPLC. Each point is the mean of duplicate determinations.

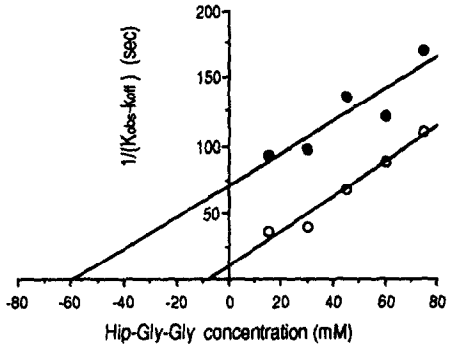

Fig. 4. Dependence of $1/(K_{obs} - k_{off})$ on Hip-Gly-Gly concentration in the presence of 50 nM idrapril (○) or 10 nM enalaprilat (●). Each point is the result of one kinetic experiment performed as reported in Fig. 2 (A and B panels), from which K_{obs} and k_{off} were determined as described in the text.

could be applied for both inhibitors to obtain the kinetic constants shown in Table 1. Direct computation of k_{off} as the y-intercept of lines in Fig. 5B gave values (2×10^{-3} and 0.58×10^{-3} sec^{-1} for enalaprilat) close to those derived from the equation in Table 1.

Discussion

As far as we know, the relative role of the two homologous and catalytically active domains of pulmonary ACE in the cleavage of Hip-Gly-Gly was not studied previously. The experiment of active site titration with lisinopril showed that just one site was mostly involved in the Hip-Gly-Gly hydrolysis. This site is assumed to be the one located in the carboxyl terminus domain, liable for the hydrolysis of other angiotensin I-related substrates [9], since Hip-Gly-Gly is also a good substrate for ACE in testis homogenate (results not shown), the isozyme containing only that domain. As a consequence, inhibition data obtained with this substrate can reasonably be considered to concern only one of the active sites in pulmonary ACE.

Under these premises, data provided in this paper establish that idrapril is a slow- and tight-binding competitive ACE inhibitor, analogous to what was reported previously for the ACE inhibitors captopril and enalaprilat

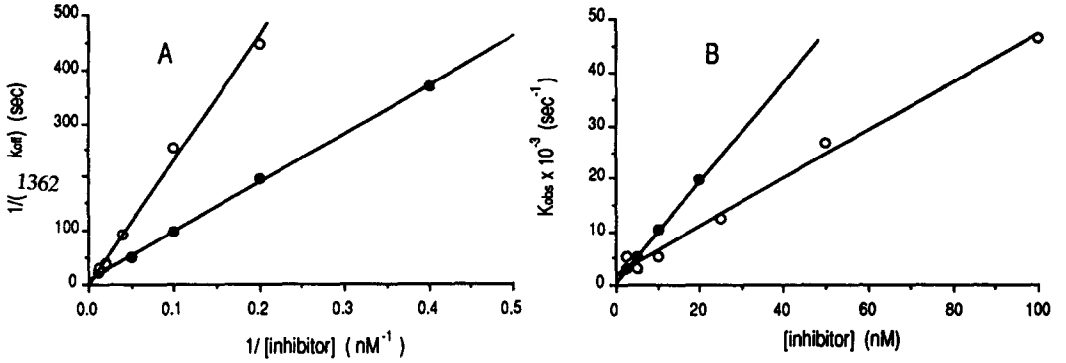

Fig. 5. Representative plots of (A) the dependence of $1/(K_{obs} - k_{off})$ on the reciprocal of idrapril (○) and enalaprilat (●) concentration and (B) the dependence of K_{obs} on inhibitor concentration, at 30 mM Hip-Gly-Gly. Each point is the result of one kinetic experiment performed as reported in Fig. 2 (C and D panels), from which K_{obs} and k_{off} were determined as described in the text.

Table 1. Rate and equilibrium constants for ACE inhibition by idrapril and enalaprilat according to mechanism A

Inhibitor	k_{off} (sec^{-1})	k_{on} ($M^{-1}\,sec^{-1}$)	K_i (pM)	Dissociation half-life (min)
Idrapril	$1.4 \pm 0.3 \times 10^{-3}$ (N = 22)	$3.0 \pm 1.5 \times 10^{6}$ (N = 4)	470	8
Enalaprilat	$0.4 \pm 0.2 \times 10^{-3}$ (N = 13)	$6.7 \pm 0.4 \times 10^{6}$ (N = 4)	61	28

Mean values (± SD) of k_{off}, calculated as the ratio $K_{obs} \cdot V_s/V_o$ and k_{on}, calculated from the slope of Eqn 2, are reported.

K_i values were calculated as the ratio of mean k_{off} and k_{on}. Half-lives were computed as $\ln 2/k_{off}$.

[3], ramiprilat [4] and lisinopril [5] in studies performed using the substrate 2-furanacryloyl-L-phenylalanyl-L-glycine (Fa-PGG). However, contrary to the generally accepted view for those compounds to interact with ACE through a multistage process (mechanism B) [13], all the analyses performed on the pre-steady-state kinetics of inhibition of Hip-Gly-Gly cleavage consistently indicated mechanism A as the most appropriate for idrapril. This seems to be confirmed by the fact that decreasing NaCl concentration, a manoeuvre which should have slowed the isomerization of EI to EI* [3], did not change the kinetics of idrapril inhibition. On the other hand, it increased the value of K_i, calculated by applying Eqn 2, to 4 nM (data not shown), due to the weakening of the positive effect of chloride on affinity of ACE for inhibitors [3].

In the presence of 300 mM NaCl, the kinetic K_i of idrapril (470 pM) was lower than that calculated by the classical steady-state approach and was comparable to that reported for captopril (330 pM) [3]. The half-life of the ACE–idrapril complex was found to be 8 min, a value quite similar to that reported for captopril (9 min) [5]. Analogies in kinetic characteristics of idrapril and captopril may well reflect the similar activity profiles which have been found when comparing the pharmacological effects of the two inhibitors [2]. Data obtained with enalaprilat in this study are in general agreement with literature data indicating mechanism B as the favourite for this inhibitor. The apparent contradictory evidence from the plot in Fig. 4B might be explained by considering that enalaprilat concentrations, used in the experiments which generated that plot, were below the value of K_i (the dissociation constant for the first EI complex). In fact, Eqn 3 tends to degenerate into the linear form of Eqn 2 when [I] becomes negligible relative to K_i. This was also observed with enalaprilat by Bull *et al.* [5], who considered mechanism A a fairly good approach to the kinetics of this inhibitor, in the absence of sound evidence for the more complex mechanism B. According to mechanism A, they calculated a K_i of 130 pM, in reasonable accordance with that obtained by us (61 pM). On the other hand, Shapiro and Riordan [3] reported K_i to be 50 pM for mechanism B, again close to the value (60 pM) estimated by us for the same mechanism. In summary, idrapril, a novel ACE inhibitor with unique chemical features, but pharmacologically similar to captopril, competitively binds to the rabbit enzyme with slow and tight kinetics. Inhibition of the hydrolysis of Hip-Gly-Gly was found to be via a single-step mechanism, at variance with what was found for enalaprilat. Considering that the ACE inhibition is known to be substrate dependent and that idrapril belongs to a structurally new class of ACE inhibitors, further studies are in progress to define better the kinetic properties of idrapril towards other substrates, with particular attention towards the physiological ones.

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Department of Pharmacology
Laboratori Guidotti SpA†
Via Livornese 402
1-56122 San Piero a Grado
Pisa
Italy

ANNALISA LIPPI*
MARCO CRISCUOLI
GIULIANA SARDELLI
ALESSANDRO SUBISSI

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* Corresponding author. Tel. (39) 50/960061; FAX (39) 50/960443.

† Company related to "A. Menarini—Industrie Farmaceutiche Riunite Srl".