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Rifamycins as inhibitors of collagenase activity: their possible pharmacological role in collagen degradative diseases

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Abstract

The interaction of commercial rifamycin SV, rifamycin B, rifampicin and some other semi-synthetic analogous with collagenase in vitro was studied by using the fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2. Increased fluorescence, due to the removal of the NH_2-terminal dinitrophenyl group, after cleavage of the probe by collagenase was monitored and related to the enzyme activity. The peptide was an efficient substrate for collagenase with a K_m of 0.83 μM at 20°C in the presence of 10 μM Ca^{2+}, pH = 7.5. In the same conditions, in the presence of rifamycin SV, rifampicin and rifamycin B or their semi-synthetic analogues AM_{41}, MC_{11} and MC_{30}, a marked inhibition of the enzyme activity was observed and an IC_{50} ranging from 13.1 to 20.7 μM was calculated. The inhibitory effect was reversible as demonstrated by restoration of enzyme activity by dialysis. These results may support the hypothesis that drugs based upon inhibitors of collagenase activity will provide a therapeutical tool for the underlying pathological destruction of extracellular matrix observed for instance in rheumatoid arthritis as reported by several authors.

Keywords: Rifamycins; Collagenase inhibition; Fluorimetric assay

1. Introduction

It has been hypothesized that in degradative diseases, such as rheumatoid arthritis (RA) and ankylosing spondylitis, the effectiveness of an active drug is dependent on one or more of the

Abbreviations: DMSO, dimethyl sulfoxide; Mca-substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2; MMP, matrix metalloproteinases; R1, rifamycin SV; R2, rifampicin; R3, AM_{41}; R4, MC_{11}; R5, MC_{30}; R6, rifamycin B; RA, rheumatoid arthritis; Ras, rifamycin antibiotics.

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following factors: reduction of activated cells of the immune system (Gabriel et al., 1990), inhibition of degradative enzyme activity, such as the matrix metalloproteinases (MMP), either modulating the proenzyme production, or activating their endogenous inhibitors, or acting itself as an exogenous modulator of MMP (Manicourt et al., 1995).

The MMP are a homologous group of zinc enzymes, active at neutral pH and requiring Ca^{2+} ions for activity, which participate in the breakdown of the major protein components of the extracellular matrix. Five major MMP have been identified in humans and implicated in connective tissue turnover and destruction. These include the fibroblast-type and neutrophyl-type interstitial collagenase that hydrolyze the type I, II and III collagen that make up the majority of the matrix (Netzel-Arnett et al., 1991). The MMP family has been implicated in a variety of diseases, including arthritis, periodontal disease and tumor cell invasion and metastasis (Nagase et al., 1994).

Collagenase, gelatinase and stromelysin belong to a family of neutral MMP which are responsible for the degradation of connective tissue components (Stack and Gray, 1989). Irreparable degradation of the extracellular matrix is a hallmark of rheumatic disease as well as in arthritic disease, in which collagenase and stromelysin-1 play an important role. (Mc Cachren, 1991; Vincenti et al., 1994). In particular interstitial collagenase or MMP-1 is found in elevated amount in the synovial joint fluid of patients with RA, and consequently, it was implicated in the process that leads to the progressive destruction of joints which take place in the disease (Hayakawa et al., 1991; Clark et al., 1993) and the level of enzyme activity correlates with the severity of the lesion (Dean et al., 1989; Clark et al., 1993).

Since collagenase plays such a fundamental role in the pathophysiology of rheumatic disease, and because the connective tissue destruction it causes is largely irreversible, its inhibition would seem to be of utmost importance in designing effective therapeutic strategies either inhibiting the enzyme activity or enzyme synthesis (Vincenti et al., 1994).

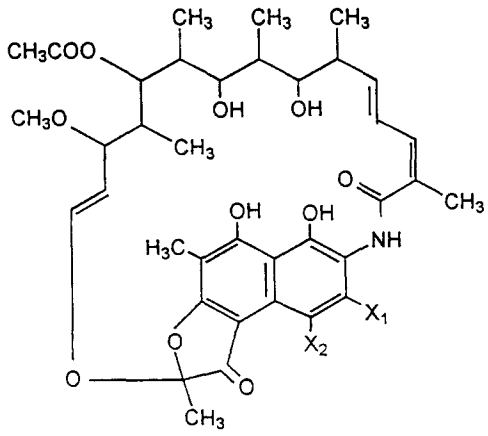
Recently it was reported on the therapeutical action exerted by rifamycin SV, administered by the intra-articular route in patients with juvenile RA. The treatment with this rifamycin resulted in a sensible regression of general features of the disease (Caruso et al., 1992, 1993), even if the mechanism of action is unclear.

In view of the central role played by collagenase in both normal and pathological catabolism of the extracellular matrix, it is crucial to understand if the activity of this MMP is affected by rifamycin antibiotics (Ras). In this context, the present study was focused on the interaction between some rifamycins, natural or chemically new semi-synthesized (Bartolucci et al., 1996), and bacterial collagenase. To this purpose we performed fluorimetric assays using as probe the synthetic esapeptide, substrate for MMP (Knight et al., 1992). The cleavage of the peptide by collagenase (Barret et al., 1989) removes the internal quencher of the probe making the product highly fluorescent. Thus, following the fluorescence signal, we observed both the inhibitory potency of six Ras and the restoration of the collagenase activity after removal of the drugs by dialysis.

2. Materials and methods

2.1. Chemicals and instrumentation

Mca - Pro - Leu - Gly - Leu - Dpa - Ala - Arg - NH_2 (Mca-Substrate), Mca-Pro-Leu-Gly (standard) were purchased from Peptide Institute, INC. (Osaka-JAPAN), Collagenase Type XI from *Clostridium histolyticum* (4.0 U/mg solid) and polyoxethylene 23 lauryl ether (Brij 35) were from Sigma Chemical Co. (St. Louis, MO, USA), Tris (hydroxy-methyl)-aminomethane was from Fluka Chemie AG (Buchs, Switzerland), Dimethyl sulfoxide (DMSO) and all other chemicals, of reagent grade, were from Merck (Darmstadt, Gemany). Rifamycin SV (R1), rifampin (R2) and rifamycin B (R6) were from commercial sources, AM_{41} (R3), MC_{11} (R4) and MC_{30} (R5) were semi-synthesized in the Institute of Structural



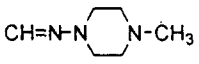
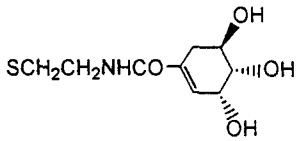
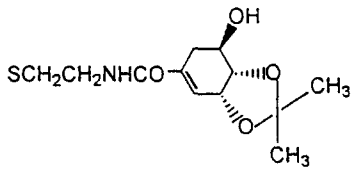
Compound		X_1	X_2
R 1	Rifamycin SV	H	OH
R 2	Rifampicin		OH
R 3	AM 41	SCH_2CH_2COOH	OH
R 4	MC 11		OH
R 5	MC 30		OH
R 6	Rifamycin B	H	OCH_2COOH

Fig. 1. Structures of the rifamycin tested antibiotics.

Chemistry of the Italian National Research Council (CNR, Rome, Italy) (Fig. 1).

The measurements were performed by a Perkin-Elmer LS-50B luminescence spectrometer computer controlled by the Fluorescence Data Manager Software (Perkin-Elmer, Bucks, England). Wavelengths setting was as follows: excitation 328 nm (slit 2.5 nm) and emission 393 nm (slit 5.0 nm).

All experiments were carried out in 0.1 M

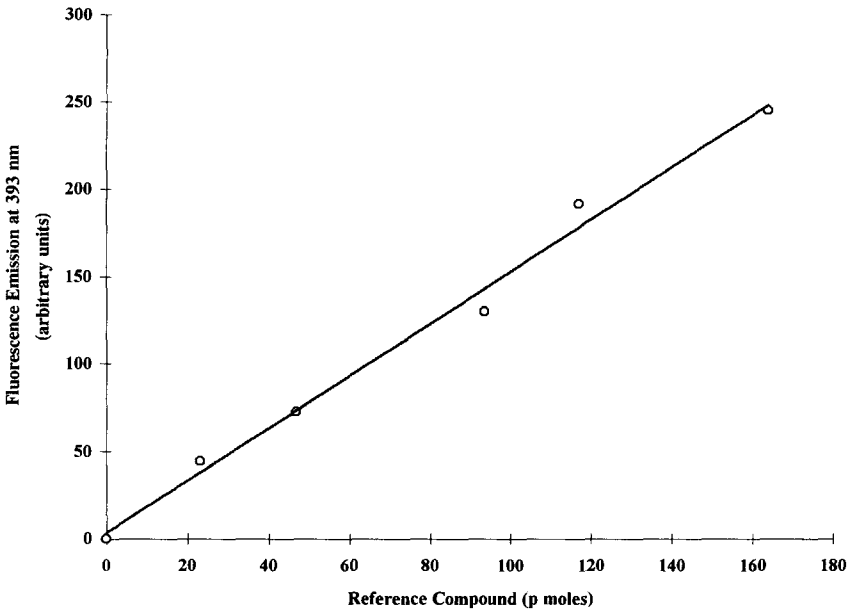


Fig. 2. Standard curve of the Mca-Pro-Leu-Gly reference compound. The points represent the mean of three independent determations with S.D. < 2%. The curve was determined by linear regression analysis. For data determination and experimental procedure see Section 2.

Tris/HCl buffer, pH = 7.5, containing 0.1 M NaCl, 10 mM $CaCl_2$, 0,05% Brij 35 and 0.02% NaN_3, referred to in the text as Tris-buffer.

2.2. Standard curve

The reference curve was constructed by using the standard solution obtained dissolving 0.11 mg of Mca-Pro-Leu-Gly (MW = 501.54) in 2.2 ml of DMSO in order to have a 0.1 mM stock solution. This solution was diluted in cuvette with Tris-buffer. The final concentrations were in the range between 7.8 and 125 nM (i.e. from 23.4 to 375.0 pmol of the reference compound).

Three independent determinations were performed on each of the diluted solution and the mean ± S.D. of these was reported in the standard curve (Fig. 2). From the curve we extrapolated the factor 0.70 by which we calculated the pmol of hydrolyzed substrate from the emission fluorescence units recorded.

2.3. Enzyme activity measurements

The Mca-substrate was previously dissolved in DMSO to a final 5 μM stock solution and stored frozen in small aliquots. The aliquots were diluted in Tris-buffer before the experiments. The collagenase solution 1 mg/ml (i.e. 4.0 U/ml) in Tris-buffer was freshly prepared. The 10^{-3} M stock solution of the inhibitors were prepared as follows: 100 μl of DMSO were added for each mg of weighted powder, successively, Tris-buffer was added to reach the final volume. No turbidity in the solution was observed.

The experiments were carried out by incubating, for 5 min at 20°C, 100 μl of diluted substrate (final concentration in cuvette 167 nM) with 100 μl (0.4 U) of the enzyme solution in Tris-buffer (in the inhibition tests various concentrations of the antibiotics, from 0.25 to 40.0 μM, were also present). After the incubation the fluorescence signal was monitored recording the signal for 3 min.

The extent of substrate hydrolysis was calculated multiplying the fluorescence values by the factor 0.70 as derived from the standard curve. The K_m was determined over a substrate concentration range of 40–350 μM and calculated by Lineweaver-Burk double-reciprocal plot. The synthetic substrate was used to determine inhibitory potency of the antibiotics (Fig. 1) added at zero time in the mixture. The value of enzyme inhibition, at each antibiotic concentration, was expressed as percent of residual activity with respect to the control. The percent of inhibition was then plotted against Ras concentrations and from the plots we determined graphically the IC_{50} of the compounds (Fig. 3). The apparent inhibition constants (K_i) were also extrapolated graphically assuming a competition between the Mca-substrate and the Ras. Under this conditions we applied the following formula:

$$v_o/v_i = 1 + K_m[I]/(K_m + [S])K_i$$

where v_o and v_i are the initial hydrolysis rate of the Mca-substrate in the absence and in the presence of inhibitors, respectively; $[I]$ is the Ras concentration and K_m and $[S]$ are the Michaelis constant and the fixed substrate concentration. The plot v_o/v_i versus the various Ras concentrations yielded a line having as slope $K_m/(K_m + [S])$ K_i from which we calculated the K_i values, see Fig. 3.

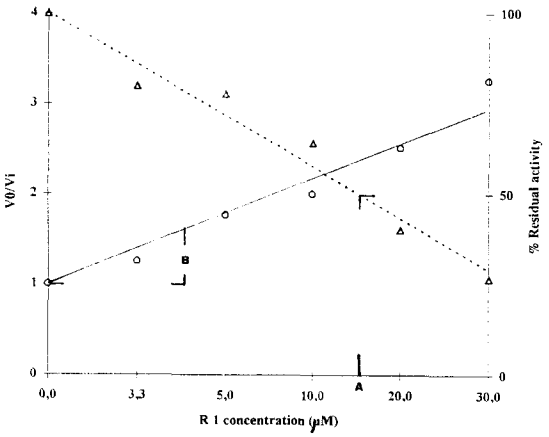


Fig. 3. Residual enzyme activity and V_o/V_i versus rifamycin contentrations. The percent of residual collagenase activity at different R1 concentrations (open triangles) represents the mean of four independent determinations (S.D. < 4%). The IC_{50} (A) was extrapolated graphically from this plot as shown in the figure. The ratio between initial hydrolysis rate in the absence (V_o) and in the presence (V_i) of rifamycins at different R1 concentrations (open circles) represents the mean of three determinations (S.D. < 6%). The slope (B) was extrapolated graphically as shown and used for the determination of K_i (see Section 2). Both curves were obtained by linear regression analysis.

2.4. Reactivation experiments

Collagenase was incubated for 15 min at 20°C with two different concentrations of each inhibitory compound (i.e. 5 and 15 μM for R2, R4 and R5; 5 and 20 μM for R1, R3 and R6), and the mixtures successively divided in two aliquots. The first was immediately tested for the inhibitory potency before dialysis, whereas the second one was dialysed for 24 h at 5°C against Tris-buffer and then measured for the reversibility effect. The pre- and post-dialysis tests were performed using a constant substrate concentration of 167 nM.

2.5. Statistical analysis

Results were expressed as mean values ± S.D. of three to five experiments and the data were statistically analysed by Student's t-test, P values below 0.05 being considered as significant. The K_m and the conversion factor for the substrate analysis, as well as the concentration causing half-maximal inhibition, indicated as IC_{50}s and K_is, respectively, were obtained from the linear regression analysis of the plots relative to the six Ras.

3. Results and discussion

Starting from the literature evidences on the therapeutical efficiency of rifamycin SV in the local treatment of juvenile RA reported by Caruso et al. (1992, 1993), we undertook an in vitro study in order to investigate the effect of this drug and its analogues on collagenase activity, since collagenase has a premier role in the irreversible degradation of the extracellular matrix seen in rheumatic diseases (Vincenti et al., 1994). The effect exerted by these rifamycins on collage-

Table 1
Inhibition parameters of rifamycins on collagenase activity

Compound	R1	R2	R3	R4	R5	R6
K_i (μM)	8.1	7.6	N.D.	8.0	8.0	11.1
IC_{50} (μM)	17.8	13.1	20.7	17.2	16.5	18.0

Assays were performed at 20°C in Tris-buffer pH = 7.5 using fixed, collagenase (0.4 U) and Mca-substrate (167 nM), concentrations. The compounds were tested at five different concentrations ranging from 40 to 350 nM, each point was in triplicate.
N.D., not determined.

nase was tested both in terms of inhibition potency and reversibility of the effect.

The study was performed fluorometrically using as substrate the synthetic esapeptide having the following sequence Mca-Pro-Leu-Gly + Leu-Dpa-Ala-Arg-NH_2, in which the symbol '+' represents the scissile bond for collagenase (Stack and Gray, 1989), Mca is the high fluorescent (7-methoxycoumarin-4-yl)acetyl and Dpa is the internal quenching group N^3-Dnp-L-2,3-diaminopropionic acid as described by Knight et al. (1992).

The cleavage removes the internal quencher (Barret et al., 1989), thus the increased fluorescence might be related to the enzyme activity using the conversion factor of 0.70 to transform the fluorescence signal (in arbitrary units) to pmol of cleaved substrate. This conversion factor was calculated from a standard curve fluorescence signal versus increasing amounts of the reference fluorescent tripeptide Mca-Pro-Leu-Gly-NH_2 (Fig. 2). Using this approach we were able to determine in our experimental conditions (i.e. type XI collagenase and 20°C) a K_m for the esapeptide substrate of 0.83 ± 0.09 μM.

Maintaining constant both the substrate (0.167 μM) and the enzyme 0.4 U and incubating for 5 min at 20°C with different concentrations of each inhibitory compound, we determined for the six (from R1 to R6) rifamycins the effect on the enzyme activity. Our assays were made with substrate concentrations well below K_m in order to reduce absorptive quencher effect as suggested by Knight et al. (1992). Plotting the enzyme activity versus the rifamycins concentrations we extrapolate the inhibitory concentration diminishing the enzyme activity by 50% (IC_{50}) and also graphically, see Section 2; we extrapolate the apparent inhibition constant (K_i). An example of this plots referring to R1 is shown (Fig. 3).

In Table 1 the calculated IC_{50} and K_i for each compound are reported. It can be seen that all the compounds tested gave similar IC_{50} values ranging from 13.1 to 20.7 μM with an apparent K_i always ten times higher than the K_m indicating that the central nucleus of the Ras rather than the lateral replacement of substituents is more important in determining the inhibition potency, even if the R1, R3 and R6, with a smaller X_1 substituent, seem to show a lower inhibition activity (i.e. higher both IC_{50} and apparent K_i) than the others, although the differences are not statistically relevant.

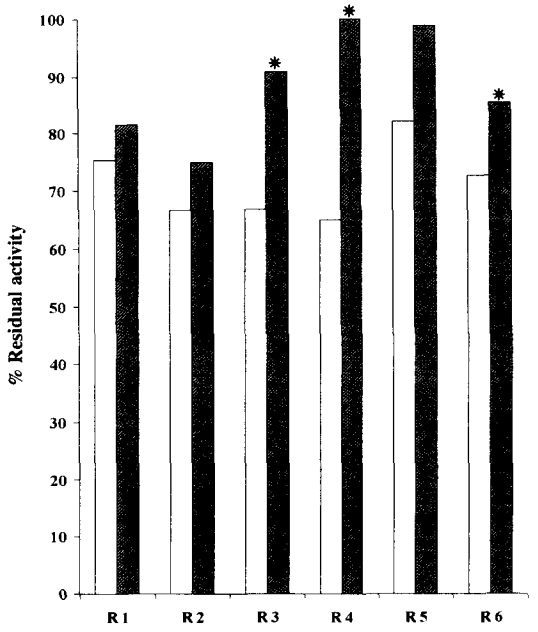


Fig. 4. Reactivation by dialysis of collagenase pre-incubated with the Ras. Open bars, enzyme activity before dialysis; hatched bars, enzyme activity after dialysis. The dialysis was initiated 15 min after the inhibitors (5 μM) were added to collagenase type XI solution (0.4 U/ml) and was carried out at 4°C against Tris-buffer. Activity was expressed as percent of control (i.e. collagenase incubated in the same conditions as the samples in absence of ihibitors). Data represent the mean of separate experiments $N = 3–5$ (S.D. < 6%). * $P < 0.05$ as determined by Student's t-test.

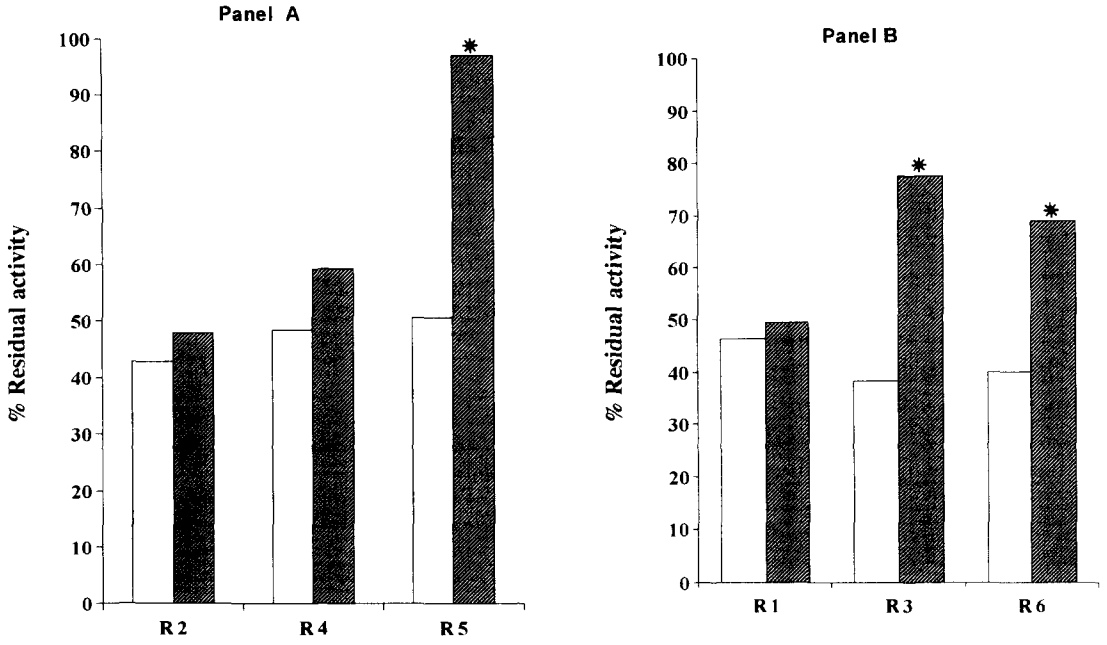


Fig. 5. Reactivation by dialysis of collagenase inhibited about 50%. Open bars, enzyme activity before dialysis; hatched bars, enzyme activity after dialysis. R2, R4 and Rs (15 μM) panel A or R1, R3 and R6 (20 μM) panel B. In the same experimental and calculation conditions as in Fig. 4. * $P < 0.05$ as determined by Student's t-test.

The presence of the Ras, all able to absorb light at 328 nm, the excitation wavelength of the Mca probe, which provoke an absorptive interference, prevented the calculation of more accurate kinetic parameters from our inhibition data. Nevertheless, control tests performed in the presence of Ras, in which increasing enzyme amounts, as well as increasing standard concentrations, were used, gave linear correlations between enzyme concentration versus its activity and standard concentration versus fluorescence intensities, respectively (data not shown), demonstrating that the spectral interference due to the inhibitors do not invalidate the test.

As far as the restoration of the activity concerns, larger variability was recorded among reversibility data collected after dialysis. The reversibility of the inhibitory effect was re-assayed after dialysing the samples for 24 h at 4°C against Tris-buffer. Each compound was tested at two different concentrations the first well below the respective IC_{50} and identical for the six Ras of 5 μM (Fig. 4) the second close to IC_{50} (i.e. 15 μM for R1, R3 and R6 or 20 μM for R2, R4 and R6), (Fig. 5, panels A and B, respectively). In all the cases a degree of activity was recovered although to a different extent. In particular this recovering was higher, presumably, when low Ras concentrations were used and in the case of R4 and R5 the full enzyme potency was recorded after dialysis (Fig. 4). It can be underlined that, after preincubation with the natural R1 and R2 the restoration of activity was always lower compared with the other Ras tested. This feature seems to indicate that the semi-synthetic Ras and R6, more hydrosoluble, although may reach the target enzyme faster than the natural rifamycins, can be removed faster thus their action seems to be less durable.

Taken together our observations in vitro with Ras and previous ones concerning the therapeutic role in vivo exerted by rifamycin SV, administered by the intra-articular route in patients with juvenile RA reported elsewhere (Gabriel et al., 1990; Caruso et al., 1992, 1993), one can argue a

possible mechanism of action of rifamycins which imply their exogenous, in this case negative, modulation of MMP as reported by Manicourt et al. (1995) for an efficient therapeutical drug in connective degradation diseases. This negative modulation, then, leads to a decreased ability by collagenase in destruction of joints, which takes place in diseases like RA (Hayakawa et al., 1991; Clark et al., 1993), and thus possible regression of the damage. This mechanism might be concomitant or alternative to the previously reported, in which on the basis of a reduction of activated cells of the immune system following to the intra-articular injection of rifamycin in arthritic patients, postulated a distal systemic effect of Ras via a mechanism of downregulation of the immune system (Caruso et al., 1993) with subsequent reduction of neutrophils. This results in a decreased MMP production and tissue destruction is, then, halted (Cawston et al., 1989).

A behaviour similar to that reported here for Ras was also postulated for other chemotherapeutic agents among which tetracyclines, IC_{50} ranging from 15 to 350 μM (Golub et al., 1991) and anthracyclines, IC_{50} ranging from 37 to 90 μM, (Karakiulakis et al., 1990) which showed MMP inhibitory properties also independent on their antimicrobial activity. However, in both cases, the reported IC_{50} were higher than that calculated for Ras either natural molecules or semi-synthetic derivatives.

Although collagenase has a premier role in the irreversible degradation of the extracellular matrix seen in RA, this last mechanism represents a complicated set of clinical disorders. As knowledge of the basic mechanisms correlated to this pathology increases, new therapeutical possibilities take place but nevertheless a successful inhibition of MMP as those exerted by Ras remains among them.

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