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# A zinc chelator inhibiting gelatinases exerts potent in vitro anti-invasive effects

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**Abstract**

Matrix metalloproteinases are zinc metalloenzymes involved in remodelling of the extracellular matrix. We compared the anti-invasive properties of a zinc ejector matrix metalloproteinase inhibitor with those of reference compounds (hydroxamic acid-based BB-94 and Ro-31-9790) which form inactive ternary complexes with the enzymes and the catalytic zinc. We show that the compound undecadenedioic acid bis-{[2-(3*H*-imidazol-4-yl)-ethyl]-amide} (S 30372) is active against gelatinases, chelates zinc and exhibits enzymatic features compatible with the potential to extract zinc from gelatinases. We then used five invasive cell lines in the Matrigel invasion chamber assay (NIH-3T3 fibroblasts, Lewis lung carcinoma cells, EJ138 and J82 bladder carcinoma and HT1080 fibrosarcoma cells). With the exception of J82 cells which were unaffected by the three inhibitors, all remaining cells were substantially more sensitive to S 30372 in terms of maximal inhibition of invasion attained. This suggests that matrix metalloproteinase inhibitors with zinc chelating/ejecting properties may be more efficient in preventing tumor progression. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Invasion; MMP-2; MMP-9; Zymography; Zinc chelation

## 1. Introduction

During the invasive metastatic process, tumor cells are confronted with the extracellular matrix of surrounding stromal tissues and with barriers such as basement membranes (Boyd, 1996). A large and growing body of evidence suggests that matrix metalloproteinases play an important role in the penetration and remodelling of these extracellular structures (Stetler-Stevenson et al., 1993; MacDougall and Matrisian, 1995). The matrix metalloproteinases form a subfamily of zinc metalloenzymes with structural similarities. They are mostly secreted as latent pro-enzymes that undergo proteolytic cleavage of an amino-terminal domain during activation and share the property of cleaving extracellular matrix components at neutral pH (Birkedal-Hansen, 1995). In particular, the MMP-2 and MMP-9 gelatinases (also called 72 and 92 kDa type IV collagenases) can degrade collagen type IV, a major component of basement membranes. The expression of these gelatinases is up-regulated during tumor invasion and metastasis (Stetler-Stevenson et al., 1993; Himelstein et al., 1994; MacDougall and Matrisian, 1995).

Specific physiological cellular inhibitors or tissue inhibitor of metalloproteinases (Murphy and Docherty, 1992; DeClerck and Imren, 1994) control the activity of matrix metalloproteinases and the balance between enzymes and inhibitors is thought to regulate the malignancy and invasiveness of cancer cells. In various in vivo models, endogenous physiological and exogenous pharmacological inhibitors of matrix metalloproteinases were shown to suppress cancer metastasis and tumor growth (Brown and Giavazzi, 1995; Conway et al., 1996; Talbot and Brown, 1996). A large group of potent inhibitors have been designed by coupling a metal-coordinating function (for example, hydroxamic acid) to a pseudo-peptidic moiety mimicking the left or right side of the collagenous cleavage site (Beeley et al., 1994). These compounds inhibit matrix metalloproteinases by forming an inactive ternary complex between the inhibitor, the enzyme and the zinc atom of the

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catalytic site. Other much less potent inhibitors of metalloenzymes are metal chelators and as such are not specific for matrix metalloproteinases. They may act by metal exclusion from the catalytic site of the enzyme. For matrix metalloproteinases, the target is a zinc atom coordinated to three histidines of the catalytic domain and present in all the enzymes studied by crystallography (Vallee and Auld, 1990; Beckett et al., 1996). The propeptide domain of the enzyme which upon cleavage permits activation provides a fourth coordination site, a cysteine (Van Wart and Birkedal-Hansen, 1990; Kleiner and Stetler-Stevenson, 1993). Interestingly, free cysteine can chelate this essential zinc atom of the matrix metalloproteinases (Stetler-Stevenson et al., 1991), although high doses are required. Similarly, other metal chelators with distinct potencies like 1-10-phenanthroline, imidazole or histidine have been shown to be inhibitors of metalloenzymes, including bacterial collagenases and other matrix metalloproteinases (Head and Yankelov, 1976; Yankelov et al., 1977, 1978; Hare et al., 1983; Makinen and Makinen, 1987).

The inhibitory effects of imidazole appear to be linked to an interaction with zinc (Yankelov et al., 1978), in a reversible monophasic manner (Springman et al., 1995). By contrast, 1-10-phenanthroline is a biphasic inhibitor of fibroblast collagenase: initially, a single molecule of chelator forms rapidly a ternary complex with zinc and the enzyme, and this step is followed by the slow removal of zinc which is subsequently chelated by a second molecule of inhibitor (Springman et al., 1995).

We were interested in comparing the cellular effects of zinc chelators or removers with those inhibitors forming inactive ternary complexes in cell invasion and proteolysis studies. Molecules with zinc-ejecting properties have previously been used for inhibition of human immunodeficiency virus (HIV-1) infectivity without causing major changes in lymphocyte cell metabolism (Rice et al., 1993). Hence, such compounds may not necessarily abrogate multiple cell functions by their metal-chelating properties.

Free histidine is a poor zinc trapper. Nevertheless, several observations raised our interest for histidine-containing agents as potential matrix metalloproteinases inhibitors with zinc removal potential. After deconvolution of combinatorial tetrapeptide libraries, the structure His–$\varepsilon$Ahx–$\beta$Ala–His was selected as a peptidic lead (Ferry et al., 1996), although having only moderate potency in the 100 $\mu$M range against gelatinases, thus being of limited interest when compared to optimized inhibitors. However, symmetrical zinc chelators containing terminal histidines have been reported to inhibit the binding of the HIV-EP1 protein to the DNA NF-$\kappa$B site by abstracting zinc from the zinc fingers of the protein (Otsuka et al., 1994). The present study reports on the action of a zinc-chelator with similar structural features: two histamine residues separated by an undecanedial linker (undecadenedioic acid bis-{[2-(3*H*-imidazol-4-yl)-ethyl]-amide} or compound S 30372). Although S 30372 is not active in the same molar range as hydroxamic acid-based matrix metalloproteinases inhibitors, the compound was found to display a more potent in vitro anti-invasive effect on various cell lines than two currently available reference inhibitors.

## 2. Materials and methods

### 2.1. Cells lines and tissue culture

NIH/3T3 fibroblast cells derived from Balb/C mice, murine Lewis lung carcinoma cells (LLC cells), human J82 vesical carcinoma cells and the human fibrosarcoma HT1080 cell line were obtained from the American tissue culture collection (ATCC, Rockville, MD); the EJ138 human bladder carcinoma cell line came from the European collection of animal cell cultures (ECACC, Salisbury, UK). Fibroblastic and tumor cells were maintained at 37°C with 5% $CO_2$–95% air in a humidified incubator in RPMI 1640 medium for LLC, 3T3 and HT1080 and in Dulbecco's Modified Eagle medium (DMEM) for EJ138 and J82, supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 10 mM HEPES, penicillin (100 U/ml) and streptomycin (100 $\mu$g/ml), all from Gibco.

### 2.2. In vitro invasion assay

The invasion assay was carried out using Transwell chambers (Costar, USA) with porous filters (pore size 8 $\mu$m) (Albini et al., 1987). Diluted Matrigel (stock solution of 10 mg/ml) was added to the porous filter (200 $\mu$l/well) and allowed to dry at 37°C for 18 h. The cells detached with 2 mM EDTA were treated subsequently or not with inhibitors, and then seeded ($2 \cdot 10^5$ cells) onto the reconstituted membrane in the upper compartment of the chamber in a final volume of 200 $\mu$l. The lower compartment was filled with 600 $\mu$l of medium with or without inhibitor and the cells were cultured at 37°C for 24 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) (5 mg/ml in phosphate buffer saline) was added in the upper (20 $\mu$l) and lower compartments (60 $\mu$l) and incubated at 37°C for 4 h. Reduction of MTT by intracellular enzymes of viable cells forms a blue formazan crystalline product (Mossmann, 1983). Invading cells were recovered on the lower side of the filter and eventually in the lower compartment. Cells on the upper surface of the filter were removed by wiping with a cotton swab. Invasion was measured spectrophotometrically at 540 nm after solubilization of the formazan product by addition of 2.5 × concentrated buffer containing SDS 50% (w/v) and dimethyl formamide/$H_2O$ 50/50 (v/v) pH 2.8 (adapted from Hansen et al., 1989) and leaving for 24 h at 37°C. The same conditions were assayed in triplicate and each assay was repeated at least twice.

### 2.3. Proliferation assay

To study the effect of inhibitors on cell growth during invasion, the cells were seeded in parallel on Matrigel in 96 well plates. After a 24-h incubation at 37°C, MTT was added to each well and optical densities were measured as described for the invasion assay. Conventional long term assays (96 well plates, four doubling-time duration) in the absence of Matrigel were also performed. Each condition was assayed in triplicate and each assay was repeated at least twice.

### 2.4. Activation of MMPs

Human purified recombinant MMP-2 and MMP-9 (from Dr. Gillian Murphy, Strangeways Laboratory, Cambridge, UK) were activated with 2 mM APMA (4-aminophenylmercuric acetate from Sigma) at 37°C for 15 min (MMP-2) or 1 h (MMP-9).

### 2.5. Fluorogenic enzymatic assay

The peptidomimetic substrate 3,5-dinitrophenyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(*N*-methylanthranilic acid)-$NH_2$ (Bachem, France) which is cleaved between amino acids Gly and Cys (Bickett et al., 1993), was used to assay the activity of the purified human recombinant MMP-2 and MMP-9. The assays were performed in 50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, 0.1% Brij35, pH 7.7 containing the activated purified MMP-2 and -9, 16 and 8 ng in each well, respectively. The reactions were started with 20 $\mu$M of substrate in a total volume of 100 $\mu$l and incubated at 37°C for 1 h. The fluorescent cleavage product was measured with a cytofluorometer (Cytofluor 2350, Millipore, France) equipped with a combination of 340 nm and 440 nm filters for excitation and emission, respectively.

### 2.6. Type IV collagen degradation assay

Collagenolytic activity was measured with $^3$H-labelled type IV collagen (NEN) (37 kbq/1 $\mu$g) as substrate (Montgomery et al., 1993). MMP-2 (8 $\mu$g/ml) or MMP-9 (4 $\mu$g/ml) and inhibitors were added to solutions containing the radiolabelled substrate (500 $\mu$g/ml) and incubated for 24 h at 37°C in 0.5 M Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij35, pH 7.5. Substrate hydrolysis was analysed by SDS PAGE (gradient 4–20%) and autoradiography. The intensity of the band corresponding to the type IV collagen alpha 2 chain was estimated by densitometry on a Pharmacia-LKB GSXL densitometer equipped with the 1D Image Master software (Pharmacia). The same conditions were assayed in triplicate and each assay was repeated three times.

### 2.7. Synthesis of inhibitors

The reference inhibitors Ro-31-9790 (Broadhurst, 1992), batimastat (Campion, 1990) and benzyloxycarbonyl-Pro-Leu-Gly-hydroxamic acid (Z-Pro-Leu-Gly-NHOH (Bickett et al., 1993) were synthesized according to published procedures. 1-10-Phenanthroline, L-histidine and histamine were purchased from Sigma.

S 30372 was synthesized as follows: histamine hydrochloride (9.7 mmol), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (9.7 mmol) and *N*-hydroxybenzotriazole (9.7 mmol) were added to a solution of undecanedioic acid (4.6 mmol) in dichloromethane (50 ml). The mixture was maintained under an argon atmosphere and *N*,*N*-diisopropylethylamine (5.15 ml, 29.6 mmol) was added dropwise The reaction was stirred at room temperature and reaction progress followed by thin-layer chromatography. After 16 h, the volatiles were removed and the residue taken up in ethyl acetate and washed with 1 M HCl, $NaHCO_3$ (5% aq.) and saturated aqueous NaCl. The organic phase was dried over $Na_2SO_4$, filtered and concentrated. Product purification was achieved using reverse phase high pressure liquid chromatography (RP-HPLC) under gradient conditions (10–40% $CH_3CN$ + 0.1% trifluoroacetic acid/30 min). The required compound provided a single peak by analytical HPLC. Nuclear magnetic resonance (NMR) analysis conformed to the appropriate structure. Elemental analysis: C: 54.00/53.79, H: 7.34/7.52, N: 18.00/17.58 (calculated/found).

### 2.8. Zinc binding studies

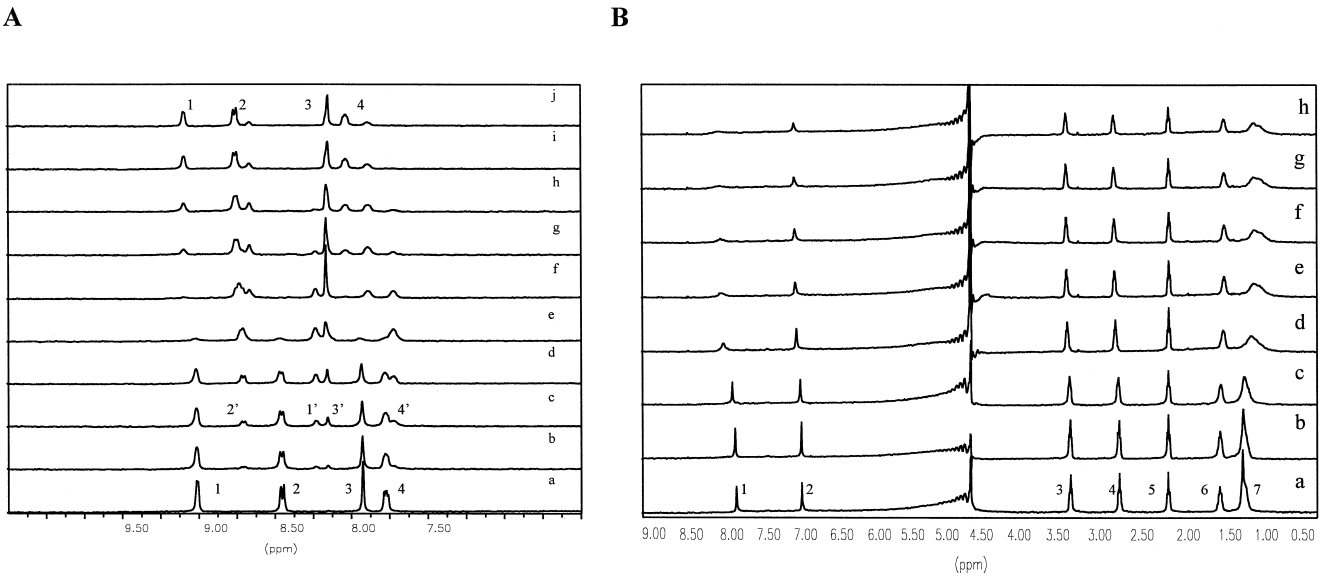
To follow the influence of $ZnCl_2$ on matrix metalloproteinases inhibitors, a series of 1D $^1$H-NMR spectra were recorded on a Bruker AM400. Reference spectra were performed in $D_2O$ and dimethyl sulfoxide (DMSO)-$d_6$ at a concentration of 9.5 $10^{-4}$ M. For each aliquot of $ZnCl_2$ added, a spectrum was recorded. $ZnCl_2$ concentrations tested ranged from 2.4 $10^{-5}$ M (first addition) to 2.3 $10^{-3}$ M (final concentration).

## 3. Results

### 3.1. Histamine-containing inhibitor of gelatinases

Previous analyses on the influence of the size of the spacer between two terminal histidines pointed to undecanedial as an optimal spacer for inhibition of gelatinases, and with a significant increase in potency compared to histidine alone or to the His–His dipeptide (Ferry et al., 1996).

Free histidine exhibited a high $IC_{50}$ in the mM range (not shown) in accordance with previous results on tissue collagenase (Yankelov et al., 1977). Interestingly, when the two amino acid histidine residues are replaced by two histamines, a further increase in inhibition potency is obtained, the corresponding molecule (S 30372, see structure in Fig. 1) being approximately 100-fold more active




## Structures

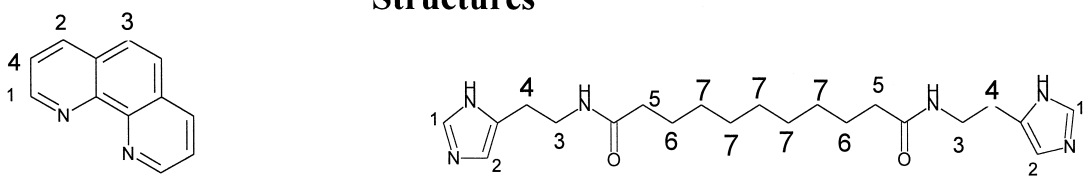


Fig. 1. [$^1$H]NMR spectra of 1-10-phenanthroline (A) and S 30372 (B) in $D_2O$ in the presence of increasing concentrations of $ZnCl_2$. Different $ZnCl_2$ concentrations (in M) are used from a to j; (a) 0, (b) $2.38 \cdot 10^{-5}$, (c) $5.96 \cdot 10^{-5}$, (d) $9.52 \cdot 10^{-5}$, (e) $1.38 \cdot 10^{-4}$, (f) $4.76 \cdot 10^{-4}$, (g) $7.15 \cdot 10^{-4}$, (h) $9.53 \cdot 10^{-4}$, (i) $1.55 \cdot 10^{-3}$, (j) $2.27 \cdot 10^{-3}$.

than histidine alone on gelatinases (Table 1). No inhibition was found with free histamine up to 5 mM (not shown). The inhibitory potential of two compounds with well-documented mechanisms of action and active in the $\mu$M range, is also presented. These compounds, 1-10-phenanthroline and Z-Pro-Leu-Gly-NHOH are representative of zinc-ejector type and ternary complex-forming inhibitors (Hare et al., 1983; Moore and Spilburg, 1986), respectively.

Although far from being as potent as optimized inhibitors such as Ro-31-9790 (Broadhurst, 1992) and batimastat (Campion, 1990), both active in the nM range (Table 1), S 30372 was further analysed in terms of its mechanism of inhibition and potential. Studies were performed at both molecular and cellular level, using hydroxamic acid-based inhibitors for comparison.

Table 1
Inhibition of peptidomimetic substrate degradation by human recombinant MMP-2 and MMP-9

| | $IC_{50}$ ($\mu$M)[a] | |
|---|---|---|
| | MMP-2 | MMP-9 |
| S 30372 | 29 | 50 |
| Ro-31-9790 | 0.0029 | 0.013 |
| Batimastat | 0.00065 | 0.00087 |
| Z-Pro-Leu-Gly-NHOH | 2 | 0.87 |
| 1-10-phenanthroline | 10.5 | 16.9 |

[a]Mean of at least three experiments carried out in triplicate (SEM less than 10%).

### 3.2. Zinc-chelating properties of S 30372

As a control for revealing zinc binding properties of the inhibitors, we used 1-10-phenanthroline. A comparison of the different $^1$H-NMR spectra recorded with successive additions of $ZnCl_2$ shows a direct interaction of zinc with 1-10-phenanthroline (Fig. 1A). As soon as $ZnCl_2$ is added (Fig. 1A, b), a new species is detected and the equilibrium is totally displaced after several additions (Fig. 1A, e). The transient coexistence of the two forms is indicative of a slow equilibrium on the NMR time scale. As the addition of $ZnCl_2$ continues, a third and a fourth species are observed, in equilibrium with the second, thus indicating that the nature of co-ordination changes. The co-ordination number could not be determined with precision. As the mixture in $D_2O$ was slightly opalescent, indicating a degree of heterogeneity, the stoichiometry of individual complexes could not be evaluated.

The same methodology was used to analyse S 30372. Comparison of the different spectra in $D_2O$ (Fig. 1B)

indicates that zinc interacts with the compound. The protons of the imidazole ring show regular changes as the $ZnCl_2$ concentration increases. In DMSO-$d_6$, observation of the exchangeable amide proton is possible. In DMSO-$d_6$, the amide protons are also sensitive to $ZnCl_2$ additions (data not shown). Moreover, in $D_2O$, some linker aliphatic protons were influenced. Relatively, the distance of aliphatic protons from the supposed interaction site is quite significant and therefore, a type of horse-shoe conformation is probably adopted by the compound—as only one species is observed—to explain their sensitivity. The interaction of S 30372 with zinc is controlled by an equilibrium shift fast on the NMR time scale.

We also evaluated the hydroxamic acid-based inhibitor Ro-31-9790 under the same conditions. Whether in $D_2O$ or in DMSO-$d_6$, no spectral changes were detected on addition of $ZnCl_2$. Hence, Ro-31-9790 shows no evidence of a direct complexation with zinc. Similarly in DMSO-$d_6$, exchangeable protons are not perturbated. Finally, UV spectra of Ro-31-9790 with and without $ZnCl_2$ did not reveal any complexation of the compound with zinc. Thus, in contrast to the hydroxamic acid-based derivative Ro-31-9790, S 30372 is a compound able to act as a zinc chelator in similarity to 1-10-phenanthroline.

### 3.3. Mechanism of inhibition by S 30372

The mechanism of inhibition of MMP-9 by given compounds was inferred by analysis of inhibition data obtained with the peptidomimetic fluorogenic substrate. Data was plotted according to the equation (Holmquist and Vallée, 1974):

$$\log(A_0/A_I - 1) = \log K_I + n \log I \quad (1)$$

wherein $A_0$ is the activity of the enzyme in the absence of inhibitor and $A_I$ is the activity of the enzyme in the presence of inhibitor at concentration $I$. It is then possible to compute the apparent number of molecules ($n$) required for inhibition: it corresponds to the slope of the graph. We included the zinc remover 1-10-phenanthroline for comparison and found a slope close to 2 (Table 2), as previously described (Moore and Spilburg, 1986; Springman et al., 1995). A slope superior to 1 indicates that inhibition occurs by removal of a catalytically important metal (Baker, 1988). The hydroxamic acid-based inhibitor [Z-Pro-Leu-Gly-NHOH] is associated with a slope close to 1, a result typical of inhibitors forming a ternary complex with the enzyme and the catalytic Zn metal (Moore and Spilburg, 1986). With the same analysis, a slope of more than 1 is found for S 30372, thus suggesting that it inhibits the enzyme by zinc removal, as observed with 1-10-phenanthroline.

Table 2
Determination of inhibitory mechanism against MMP-9

| | $n$ | Proposed mechanism |
|---|---|---|
| 1-10-phenanthroline | $1.86 \pm 0.04$ (3) | zinc removal |
| Z-Pro-Leu-Gly-NHOH | $1.13 \pm 0.01$ (2) | ternary complex formation |
| S 30372 | $1.94 \pm 0.34$ (3) | zinc removal |

Inhibition data were plotted according to Eq. (1) (see text). $n$ represents the number of moles of inhibitor bound per mole of enzyme according to Eq. (1) (see text) and the values are means ± SEM or range of the number of experiments indicated in parentheses.

### 3.4. Anti-invasive in vitro effect

We compared the effects of the two types of inhibitor on in vitro cell invasion. Five established cell lines were used: two human bladder carcinomas (J82 and EJ138), a murine Lewis lung carcinoma (LLC), a human fibrosarcoma (HT1080) and mouse 3T3 fibroblasts. All the cell lines studied were invasive in the in vitro Matrigel assay. Analyses of cytotoxicity were also performed over a period corresponding to 24 h, the duration of the invasion assay, with or without Matrigel coated on the plate. This permitted estimation of the maximal tolerated doses giving a non-significant effect on cell viability. The corresponding doses were found to be 1 mM for S 30372 and 10 $\mu$M for Ro-31-9790 and batimastat, independent of cell line. Both the zinc ejector compound, 1-10-phenanthroline, and the hydroxamic acid-based derivative, Z-Pro-Leu-Gly-NHOH, were not assayed at cellular level in the invasion assay due to their toxicity. Hence, S 30372 was tested and compared with Ro-31-9790 and batimastat.

In invasion assays with LLC, 3T3, EJ138 and HT1080 cell lines, S 30372 was more efficient than batimastat and Ro-31-9790: although much higher doses were needed to block invasion, the percentage of maximal inhibition reached at non cytotoxic doses was always higher (Fig. 2). Ro-31-9790 and batimastat reached a plateau of maximal inhibition at 5–10 $\mu$M. This plateau was always lower than the maximal inhibition obtained with compound S 30372. It is noteworthy that a plateau is reached with S 30372 when assayed against HT1080 cells, which is in contrast to the other cell lines studied. This plateau (around 35% inhibition), although still higher than with the hydroxamic acid-based inhibitors (20–25%), is far lower than the 50–70% inhibition obtained with EJ138, LLC and 3T3 cells. Finally, J82 cells were not affected by batimastat and Ro-31-9790 and were marginally inhibited by S 30372 ($15.5 \pm 2\%$ at 1 mM).

Long-term cytotoxicity assays revealed that S 30372 exhibited $IC_{50}$ values in the 500 $\mu$M range (vs. more than 1 mM during a 24-h drug exposure, see Fig. 2). In contrast, the $IC_{50}$ values of the reference compounds shifted from the 200–300 $\mu$M and 100–200 $\mu$M range at 24 h to the 30–100 $\mu$M and 10–60 $\mu$M range at 96 h, for Ro-31-9790 and batimastat, respectively. Therefore, since S 30372 exhibited cytotoxicity in the long-term with $IC_{50}$ values spanning the range of doses assayed in the 24 h

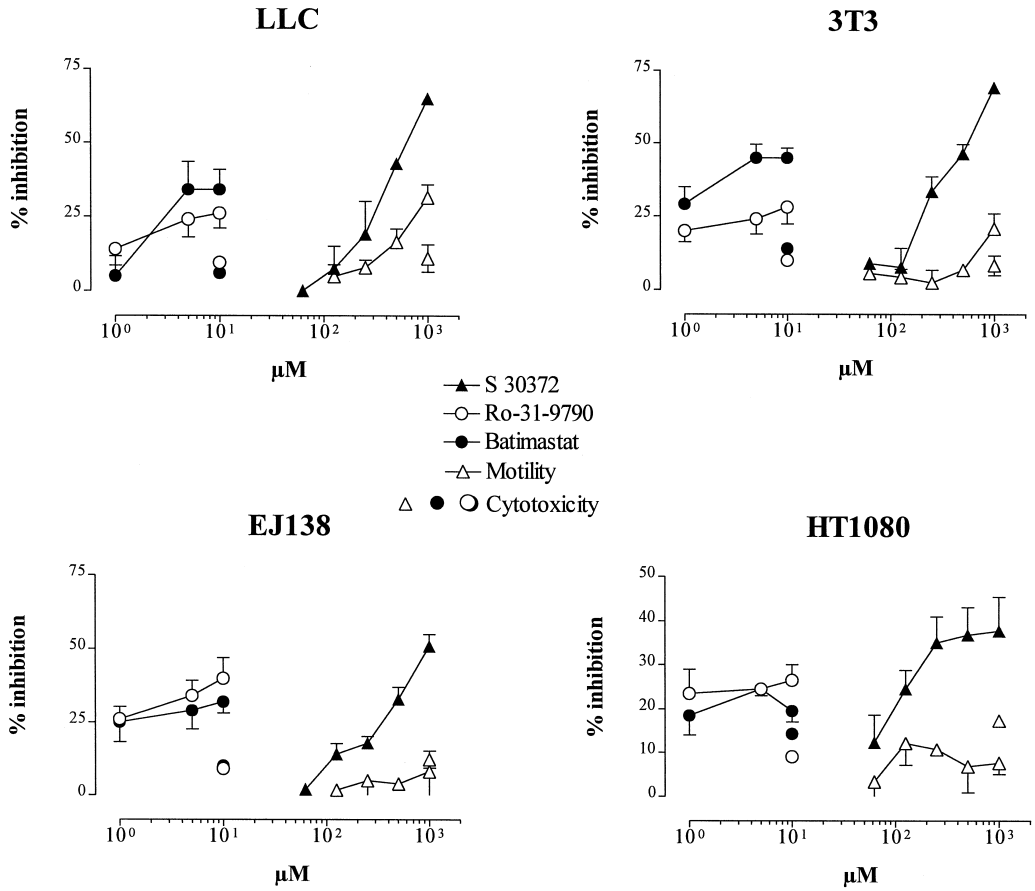



Fig. 2. Effect of S 30372, Ro-31-9790 and batimastat on the invasion of LLC, 3T3, EJ138 and HT1080 cells into a reconstituted basement membrane. Cells were seeded with various concentrations of inhibitors into the upper and lower compartments of a Transwell chamber with filters precoated with (invasion, solid lines with compound names) or without Matrigel (motility, for the S 30372 compound only). After 24 h of incubation, the number of cells which had reached the lower surface was indirectly estimated. Mean ± SEM of three experiments carried out in triplicate. The effects of each compound on cell survival at the highest doses tested are also indicated (single symbols, mean ± range of two experiments carried out in triplicate) and were derived from the 96 well plate cytotoxicity assay in the presence of Matrigel, after a 24-h drug exposure.

invasion assays, it was important to further understand the origin of the higher potency of S 30372 vs. reference compounds in invasion assays, in particular in terms of the potential contribution of cytotoxicity. For a given cell line, the maximal doses tested for the three compounds gave similar inhibition of cell survival after a 24-h exposure on Matrigel, i.e., around 10 to 15% of inhibition (Fig. 2, single symbols, and 12.4 ± 3.4%, two experiments in triplicate for J82 cells). Moreover, with the exception of LLC cells, the contribution of cell motility to the invasion process was also found to be in this order of magnitude for the S 30372 compound (Fig. 2, open triangles, and 14.6 ± 1.2%, two experiments in triplicate for J82 cells).

Finally, we verified that S 30372 could indeed inhibit the degradation of an ECM component present in Matrigel, type IV collagen. Table 3 shows that potencies similar to those obtained with the peptidomimetic substrate (Table 1) are observed when S 30372, Ro-31-9790 and batimastat are assayed in the presence of this preferential substrate of the MMP-2 and MMP-9 gelatinases.

Table 3
Inhibition of $^{3}$H-labelled type IV collagen degradation by MMP-2 and MMP-9

| μM | $IC_{50}$ (μM)[a] | |
|---|---|---|
| | MMP-2 | MMP-9 |
| S 30372 | 62 | 125 |
| Ro-31-9790 | 0.1 | 0.05 |
| Batimastat | 0.001 | 0.001 |

[a] Means of three experiments. SEM less than 10%.

## 4. Discussion

Our present objective was to compare the effect of a zinc ejector matrix metalloproteinases inhibitor on in vitro invasion with the anti-invasive properties of two hydroxamic acid-based matrix metalloproteinases inhibitor reference compounds, batimastat (BB-94) and Ro-31-9790. The molecule 1-10-phenanthroline, active in the 10 μM range against gelatinases, is the prototype of zinc ejector MMP

inhibitors (Moore and Spilburg, 1986; Springman et al., 1995), and was used as a reference compound in enzymatic assays. However, its cytotoxic profile prevented further analysis in vitro at the cellular level. The compound undecadenedioic acid bis-{[2-(3*H*-imidazol-4-yl)-ethyl]-amide} (S 30372) is active against gelatinases, although with moderate potency (29 and 50 $\mu$M IC50 against MMP-2 and -9) and exhibits features compatible with zinc ejecting potential: biochemical studies of inhibition mechanism suggest that contrary to hydroxamic acid-based inhibitors forming a ternary complex with the gelatinase and its catalytic Zn atom, S 30372 inhibits the enzyme by zinc removal in accordance with 1-10-phenanthroline. It is noteworthy that structurally-related compounds (symmetrical metal chelators with terminal histidines) indeed act as zinc ejectors (Otsuka et al., 1994). However, we cannot totally exclude that molecules of S 30372 bind to the enzyme at more than one site, a process which may also lead to a value of $n$ superior to 1 in Eq. (1) (Auld, 1988). Whatever the precise mechanism, it appears to be clearly distinct from that of hydroxamic acid-based inhibitors. Moreover, [$^1$H]NMR spectral analyses of the zinc-chelating properties of selected compounds revealed that 1-10-phenanthroline and S 30372 interact with zinc in solution, whereas the hydroxamic acid-based inhibitor Ro-31-3790 does not.

We then used five established invasive cell lines in the Matrigel invasion chamber assay, including murine NIH-3T3 fibroblasts and LLC Lewis lung carcinoma cells, human EJ138 and J82 bladder carcinoma and HT1080 fibrosarcoma cells. With the exception of J82 cells, which were unaffected by the two types of inhibitors, all the cells were more sensitive to S 30372 in terms of maximal inhibition attained. Nevertheless, at the highest doses of inhibitor tested, cell invasion was not totally blocked, possibly as a result of the presence of other enzymatic systems besides gelatinases. Indeed, the degradation of extracellular matrices involves the participation of a large ensemble of distinct enzyme families (Chauhan et al., 1991; Rochefort and Capony, 1991; Schmitt et al., 1992; MacDougall and Matrisian, 1995) and they may act independently or in a proteolytic cascade (Schmitt et al., 1992). Furthermore, the repertoire of secretion of these enzymes differs from one cell line to another. Therefore, it is not surprising that the inhibition efficiency of the three compounds tested also varies from one cell line to another.

It is notable that the S 30372 compound exhibited a much smaller ratio between its potency in cell-based assays vs. enzymatic assays than the hydroxamic acid-based derivatives (3–10 vs. 100–10 000-fold, respectively, compare activities in Tables 1 and 3 vs. Fig. 2). Since the enzymatic assays presented concerned the activity of gelatinases, this may thus possibly reflect that other matrix metalloproteinase enzymes, besides gelatinases, are targeted by the hydroxamic acid-based compounds and with higher $IC_{50}$ values. Further analyses are required to demonstrate an effective participation of gelatinases in the invasion studies presented. However, we verified that all five cell lines produce gelatinases as assayed by zymography (not shown), although no clear-cut correlation could be established, since they expressed the gelatinases to varying degrees and with differential activation.

Also, we cannot totally exclude that other proteases, besides matrix metalloproteinases, may be inhibited by S 30372 during the invasion process. For instance, as a metal chelator, it is likely to have other metalloenzymes as secondary targets, such as aminopeptidase N or CD13, a metalloenzyme reported to be involved in in vivo and in vitro invasion of HT1080 cells (Saiki et al., 1993; Fujii et al., 1996). This may account for the apparent higher inhibitory potential of S 30372 vs. hydroxamic acid-based specific matrix metalloproteinase inhibitors. Matlystatine A is an example of such an anti-invasion inhibitor, where effect is associated with a dual anti-aminopeptidase N and anti-matrix metalloproteinases potential (Fujii et al., 1996).

Another likely consequence of potential differences in molecular targets of the hydroxamic acid-based inhibitors and S 30372 is that distinct functions are targeted. Thus, as invasion is the result of a combination of extracellular matrix destruction and cellular translocation, we assayed the effects of both types of inhibitors on cell motility through uncoated filters over a 24-h period. The locomotory potential of EJ138, HT1080, and 3T3 cells was not specifically affected, whereas a 30% decrease in motility was found for LLC cells with a 1 mM solution of S 30372. Batimastat and Ro-31-9790 did not perturb specifically LLC cell translocation at the doses tested (not shown). Therefore, with the possible exception of LLC cells, it seems likely that the primary target of both types of compound is matrix proteolysis and not intrinsic cell motility. Also, the motility data and the fact that all compounds influenced cell survival to the same extent at the highest doses tested, suggest that the higher inhibitory potential of S 30372 does not reflect higher cytotoxicity. The presence of a plateau of inhibition of HT1080 invasion at several doses of S 30372 and the absence of specific effect on J82 cell invasion further strengthen this conclusion.

The absence of any significant effect of the matrix metalloproteinase inhibitors tested on J82 cells probably results from the presence of other enzymatic systems, not affected by the compounds, which are responsible for the observed invasion in vitro of this cell line. It also suggests that although gelatinases are produced, the J82 cell line does not use these enzymes for local invasion. More importantly, it suggests that the metal-chelating properties per se of the S 30372 compound or other intrinsic property not shared by the hydroxamic acid-based inhibitors and independent of matrix metalloproteinases inhibition, may not account for enhanced anti-invasive effects, since, otherwise, J82 cells would be likely to respond. On an all-or-none basis, the responses to hydroxamic acid-based inhibitors paralleled those of S 30372. Further exploration

of the activity of S 30372 or more potent derivatives on a larger panel of cell lines is required.

In conclusion, we have shown that a non-optimized metal-chelating inhibitor of gelatinases, at least 100 to 10 000-fold less active at the enzymatic level than hydroxamic acid-based inhibitors, exhibits a stronger inhibition potential on in vitro cell invasion of four cell lines. This stronger inhibitory effect is not reflected by a lower molar requirement of compound, even though the potency of the two types of inhibitors at the cellular level are separated by only a 100-fold. Additional studies are required before concluding on the importance of the putative zinc-ejection potential of S 30372 and its in vitro anti-invasive effects. However, one may consider that molecules with optimized zinc-chelating properties may well prove to be an interesting new class of matrix metalloproteinase inhibitors.

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