Bioorganic & Medicinal Chemistry 17 (2009) 1290–1296

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and biological evaluation of curcuminoid pyrazoles as new therapeutic agents in inflammatory bowel disease: Effect on matrix metalloproteinases

R. M. Claramunt [a,*], L. Bouissane [a], M. P. Cabildo [a], M. P. Cornago [a], J. Elguero [b], A. Radziwon [c], C. Medina [c,*]

[a] Departamento de Química Orgánica y Bio-Orgánica, Facultad de Ciencias, UNED, Senda del Rey 9, E-28040 Madrid, Spain
[b] Instituto de Química Médica, CSIC, Juan de la Cierva 3, E-28006 Madrid, Spain
[c] School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, 23 Westland Row, Dublin 2, Ireland

ARTICLE INFO

Article history:
Received 10 September 2008
Accepted 8 December 2008
Available online 6 January 2009

Dedicated to Professor M del Carmen Pardo of the 'Universidad Complutense de Madrid' on the occasion of her retirement

Keywords:
Curcumin
Pyrazole
Inflammatory bowel disease
Crohn's disease
Ulcerative colitis
Matrix metalloproteinases
Gelatinases
Caco-2 cells

ABSTRACT

Seven N-unsubstituted curcuminoid pyrazoles have been synthesized from the corresponding β-diketones (including curcumin). We evaluated the possibility of curcuminoid pyrazoles regulating the activity of matrix metalloproteinases (MMPs) by human intestinal epithelial cells in vitro. Zymographic analysis revealed that three compounds significantly down-regulated MMP-9 activity on inflammation-induced intestinal epithelial cells, making them original candidates for the treatment of inflammatory bowel disease (IBD).

1. Introduction

Inflammatory bowel disease (IBD), both ulcerative colitis (UC) and Crohn's disease (CD), is a chronic relapsing condition with inflammation and tissue remodeling of the gastrointestinal tract whose etiology remains unknown.[1,2] However, it is now apparent that the intestinal epithelium may play an important role in the immunomodulatory response of the intestinal mucosa. In addition, existing therapies in IBD, predominantly aminosalicylates, steroids and immunosuppressants, can relieve the inflammatory symptoms; however, IBD patients may suffer numerous relapses and there is no cure.

Matrix metalloproteinases (MMPs) comprise a group of zinc and calcium-dependent endopeptidases that are involved in the remodeling and degradation of extracellular matrix (ECM) during physiological and pathological conditions, such as tumor growth, metastasis and inflammatory reactions. Based on substrate specificity, MMPs have been classically divided into collagenases, gelatinases (MMP-9 and MMP-2) and stromelysins. Several lines of evidence support the involvement of these enzymes in accelerated breakdown of the ECM in IBD and therefore, contribute to the pathogenesis of this condition.[3–7] In fact, the gelatinase MMP-9 which is released by intestinal cells in response to pro-inflammatory cytokines such as TNF-α and IL-1β, becomes active and it is the most abundantly MMP expressed in the inflammatory tissues of IBD patients.[3] In addition, we and others have found that pharmacological inhibition of MMPs is protective in several experimental models of colitis.[8–12]

The aim of this study was to test new synthetic curcumin pyrazole derivatives **1**–**7** in intestinal inflammation. Curcumin **8** [E-100: 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the main colorant present in the rhizome of *Curcuma longa*. The root of this plant has been used in India as preservative, colorant, flavoring in meals (curry) and as a traditional medicine (one significant finding is that the four most frequent neoplasias—colon, breast, prostate and lung—are less frequent in India, where this ingredient is widely used). It is known that **8** acts as

* Corresponding authors. Tel.: +34 913987322; fax: +34 913988372 (R.M. Claramunt), tel.: +35 318962823 (C. Medina).
E-mail addresses: rclaramunt@ccia.uned.es (R.M. Claramunt), cmedinammar@hotmail.com (C. Medina).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.12.029

an antioxidant,[13–15] anti-inflammatory,[16–18] antitumoral (antiangiogenic),[16,17,19–21] and that it corrects the genetic mutation that causes cystic fibrosis,[22] which explains the growing interest in this material. Other studies of the properties of curcumin and related β-diketones (curcuminoids) include their properties as metal complexants,[23] their binding to DNA,[24] their proton transfer and tautomerism,[25] and their immunopharmacology.[26] Three recent reviews have been devoted to curcumin and the curcuminoids.[27–29] Concerning SAR, it appears that the presence of the phenolic rings is a prerequisite for activity.[14,30,31]

A way to modify the curcumin β-diketone skeleton is to transform it into an heterocycle, for instance, reacting it with hydrazines to form pyrazoles. Three strategies are possible (Scheme 1): (**i**) to react curcumin itself with different monosubstituted hydrazines (including hydrazine itself), (**ii**) to react curcuminoids (including curcumin) with hydrazine itself (compound **1** are obtained by both ways), and (**iii**) mixing both approaches. Pyrazole **1** derived directly from curcumin has been prepared many times since 1991.[32–38] It is a pale yellow solid that melts at 211–214 °C[32] or 215 °C.[34]

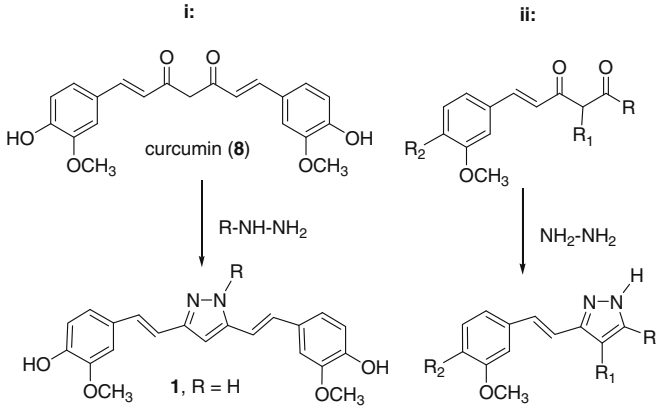
The activity of the curcuminoid pyrazoles covers domains such as anti-inflammatory (5-lipooxygenase and cyclooxygenase inhibitors),[32,36] antitumoral (anti-angiogenic),[33–35] and drugs for the treatment of the Alzheimer disease (potent γ-secretase inhibitors, potent ligands for fibrillar Ab42 aggregates, tau aggregation inhibitors, and depolymerizing agents for tau aggregates).[37,38] In the last and more recent application (type **i** approach with 11 different R including H), curcumin-derived pyrazoles were synthesized in order to minimize the metal chelation properties of curcumin. The reduced rotational freedom and the absence of stereoisomers was anticipated to enhance the inhibition of γ-secretase with lesser secondary effects. Accordingly, the replacement of the 1,3-dicarbonyl moiety by isosteric heterocycles turned these compounds very interesting candidates for AD research. Finally, it must be considered that pyrazoles, and amongst them Celecoxib, are inhibitors of MMP-9.[39–41]

We decided to explore way **ii** preparing the seven pyrazoles reported in Figure 1 with the aim of studying them in IBD.

2. Results and discussion

2.1. Chemistry

The synthesis of curcumin pyrazoles **1**–**7** was achieved following the procedure described for the preparation of the parent compound **1**[32,34] starting from the corresponding β-diketones **8**–**14** and hydrazine, as depicted in Scheme 1. All compounds have been fully characterized and the NMR assignments completed as shown in the Experimental Section.



Scheme 1. Pyrazoles related to curcumin.

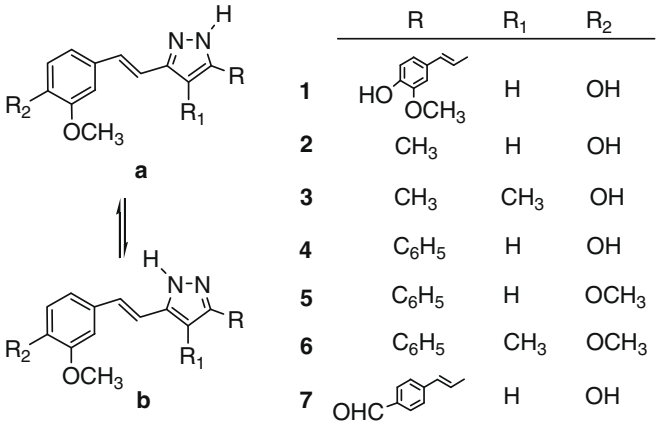


Figure 1. Pyrazoles studied as new therapeutic agents for IBD.

Discussion on the *NH*-tautomers (**a** and **b**) stability and the tautomerization equilibrium constant for each pyrazole **1**–**7** is not the object of the present paper and will be described elsewhere.[39]

2.2. Pharmacology

Knowing that intestinal epithelial cells may play an important role in the immunomodulatory response in IBD, we decided to use Caco-2 cells, human epithelial intestinal cells, in our experiments. Since MMP-9 is one of the main proteolytic enzymes involved in human IBD and TNF-α and IL-1β are pro-inflammatory cytokines that are over-expressed in this condition, we tested the effect of these cytokines on the activity of gelatinases produced by caco-2 cells. In fact, MMP-9 seems to play a main role in inflammation-associated tissue destruction both in human IBD and experimental colitis.[3,10,12,43,44] A significant correlation between the MMP-9 activity and the tissue damage has been previously found.[3,12] Indeed, either pharmacological MMP-9 inhibition or MMP-9 deficiency have been proven useful in different experimental models of colitis.[10,12,43,44] Therefore, inhibition of this enzyme in IBD seems to be an excellent therapeutic strategy.

In our experiments, we found that in untreated cells, MMP-2 was the predominant gelatinase, whereas MMP-9 was negligible as measured by zymography. However, cytokine treatment resulted in a significant dose-dependent up-regulation of MMP-9 activity in intestinal epithelial cells. Nevertheless, MMP-2 remained unchanged by the cytokine treatment (Fig. 2A and B). We corroborated these results using specific antibodies against MMP-9 (Fig. 3A and B). Again, cytokine treatment induced an up-regulation of MMP-9 expression as measured by western-blotting. These results are completely in agreement with previous studies.[44,45] In fact, gene transcription of MMP-9 is inducible and the promoter region is highly responsive to most growth factors and cytokines in several cells. In contrast, MMP-2 is expressed constitutively by most cells and appears to be moderated induced or repressed.[46,47]

Since the concentration of 10 ng/ml TNF-α and IL-1β was found to be really effective inducing the up-regulation of MMP-9 activity, further experiments were performed with this concentration of cytokines. We found that co-treatment with several compounds, such as **1**, **2** and **3** at 50 μM, resulted in a significant down-regulation of MMP-9 activity as measured by zymography (Fig. 4A and B).

To further explore the optimal concentration of the three compounds that were able to down-regulate MMP-9 activity in vitro,

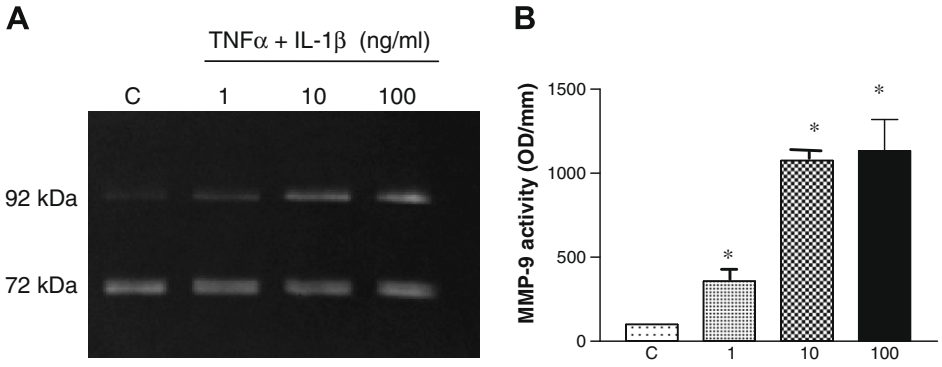


Figure 2. Activity of gelatinases in Caco-2 cells in absence (c) or presence of TNF-α and IL-1β (1, 10 and 100 ng/ml). (A) Representative zymogram of gelatinases with molecular weights of 72 and 92 kDa corresponding to MMP-2 and MMP-9, respectively. (B) Quantitative zymographic analysis ($^{*}p < 0.05$ vs control).

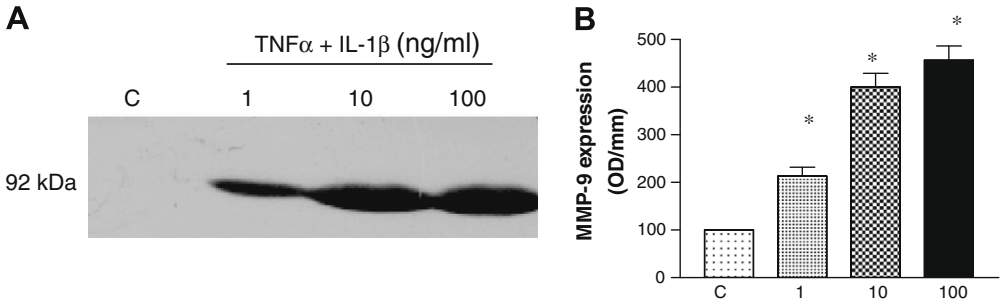


Figure 3. MMP-9 expression in Caco-2 cells in absence (c) or presence of TNF-α and IL-1β (1, 10 and 100 ng/ml). (A) Representative western-blot of MMP-9 with a molecular weight of 92 kDa. (B) Quantitative western-blot analysis ($^{*}p < 0.05$ vs control).

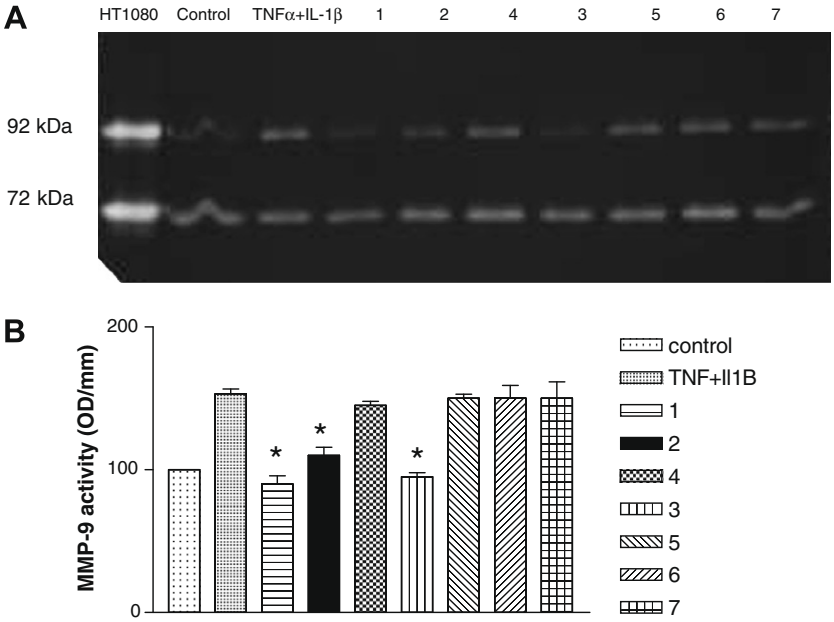


Figure 4. Activity of gelatinases in Caco-2 cells stimulated by TNFα and IL-1β (10 ng/ml) in the presence of the different curcumin pyrazoles at a concentration of 50 μM. (A) Representative zymogram of gelatinases, MMP-9 (92 kDa) and MMP-2 (72 kDa), in untreated cells (control), stimulated cells with cytokines (TNFα + IL-1β) and stimulated cells with cytokines and in the presence of the different curcumin pyrazoles (1–7). Conditioned media of HT1080 human fibrosarcoma cells which express high amount of both gelatinases were used as positive control. B: Quantitative zymographic analysis ($^{*}p < 0.05$ vs TNFα+IL-1β).

additional experiments were carried out. The dose-response curves for the most active compounds 1–3 are shown in Figure 5A–C.

Further experiments were carried out with regular curcumin as it is known that this compound down-regulates MMP-9 activity. In fact, it has been found that curcumin inhibits MMP-9 in human articular chondrocytes[48] and in blood mononuclear cells.[49] The concentration-response curve is shown in Figure 6. As expected, curcumin was able to significantly down-regulate MMP-9 activity in caco-2 cells stimulated by pro-inflammatory cytokines to the same extent than curcumin-derived pyrazoles. Therefore, curcumi-

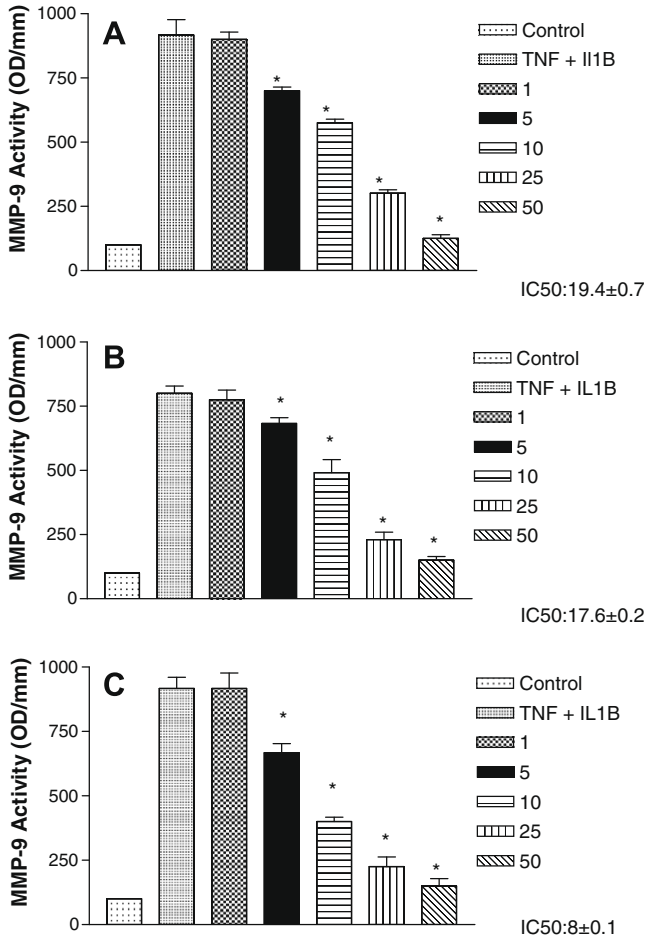


Figure 5. MMP-9 activity in Caco-2 cells stimulated by TNFα and IL-1β (10 ng/ml) in the presence of curcumin pyrazoles **1**–**3** at different concentrations (1, 5, 10, 25, 50 μM). A, B and C represents the quantitative analysis of MMP-9 activity in untreated cells (Control), stimulated cells with cytokines (TNF + IL-1) and stimulated cells with cytokines and in the presence of the curcumin pyrazoles **1** (A), **2** (B) and **3** (C) at different concentrations (1, 5, 10, 25 and 50 μM) ($^{*}p < 0.05$ vs TNFα+IL-1β) and IC50, respectively.

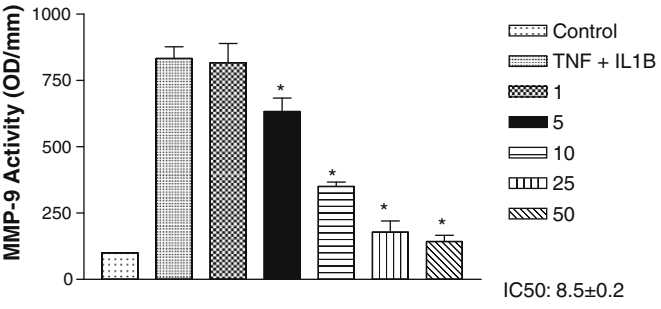


Figure 6. Quantitative analysis of MMP-9 activity in untreated cells (Control), stimulated cells with cytokines (TNF+IL-1) and stimulated cells with cytokines and in the presence of curcumin **8** at different concentrations (1, 5, 10, 25 and 50 μM) ($^{*}p < 0.05$ vs TNFα+IL-1β) and IC50.

noid pyrazoles derivatives are as effective as regular curcumin inhibiting MMP-9 activity on inflammation-induced intestinal epithelial cells. Hence, these new compounds raise the possibility of a new therapeutic approach to human IBD.

3. Conclusion

Three pyrazoles have demonstrated activity in the biological tests used as models for IBD (Scheme 2).

The main points of this study are: (i) although pyrazole **1** had already been synthesized and biologically tested, its possible application in the field of IBD is described here for the first time; (ii) two new pyrazoles **2** and **3** have shown activities similar to **1**; (iii) the fact that a 3(5)-methyl group is better than a 3(5)-phenyl group (compounds **4**–**6**) was unexpected and offers interesting possibilities of lead optimization.

Further studies are guaranteed to test the therapeutic effect and minor secondary effects of these compounds in vivo.

4. Experimental

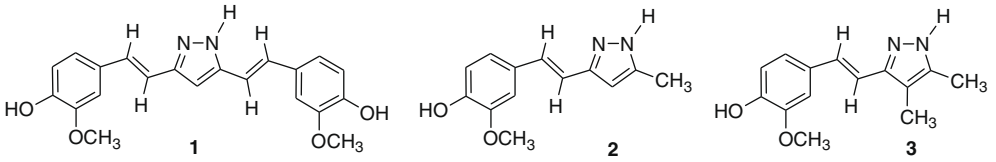
4.1. Chemical procedures

Melting points for pyrazoles **1**–**7** were determined by DSC on a Seiko DSC 220C connected to a Model SSC5200H Disk Station and for the other compounds a hot stage microscope was used. Thermograms (sample size 0.003–0.0010 g) were recorded at the scanning rate of 2.0 °C min^{-1}. Thin-layer chromatography (TLC) was performed with Merck silica gel (60 F_{254}). Compounds were detected with a 254-nm UV lamp. Silica gel (60–320 mesh) was employed for routine column chromatography separations. Elemental analyses for carbon, hydrogen, and nitrogen were carried out by the Microanalytical Service of the 'Universidad Complutense de Madrid' on a Perkin–Elmer 240 analyzer. The NMR spectra were recorded on a Bruker DRX 400 (9.4 T, 400.13 MHz for ^{1}H, and 100.62 MHz for ^{13}C) spectrometer with a 5-mm inverse detection H-X probe equipped with a *z*-gradient coil at 300 K. Chemical shifts (δ in ppm) are given from internal solvent, for ^{1}H, $CDCl_3$ (7.26), [D_6]DMSO (2.49), for ^{13}C, $CDCl_3$ (77.0), [D_6]DMSO (39.5). 2D (^{1}H–^{1}H) gs-COSY and inverse proton detected heteronuclear shift correlation spectra, (^{1}H–^{13}C) gs-HMQC, and (^{1}H–^{13}C) gs-HMBC were acquired and processed using standard Bruker NMR software and in non-phase-sensitive mode. [50] Commercial curcumin or (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (**8**) was used after purification and its NMR data have already been published.[51]

4.1.1. General procedure for the preparation of pyrazole derivatives (1–7)

Compounds **1**–**7** were prepared by reacting the corresponding β-diketones **8**–**14** (1 mmol) with hydrazine hydrate 98% (1.5 mmol) in acetic acid (5 mL). After heating at reflux for 2 h the reaction mixture was poured into water. The precipitate was filtered off, washed with water and dried. The solid was purified by column chromatography using ethyl acetate as eluent. The NMR atom numbering for pyrazoles **1**–**7** is reported in Scheme 3.

4.1.1.1. (*E*)-3,5-Bis[β-(4-Hydroxy-3-methoxyphenyl)-ethenyl]-1*H*-pyrazole (1). Prepared from purified commercially available curcumin (**8**). The compound was recrystallized from H_2O–EtOH to give a colorless solid (1 g, 2.74 mmol, 63%). Mp 217.1 °C, lit. 211–214 °C,[32] 215 °C.[34] Anal. Calcd for $C_{21}H_{20}N_2O_4$ (364.14): C, 69.22; H, 5.53; N, 7.69. Found: C, 68.79; H, 5.53; N, 7.70. ^{1}H NMR ([D_6]DMSO): δ = 3.82 (s, 3H, 3′-OCH_3), 6.61 (s, 1H, H-4), 6.76 (d, J_3 = 7.5 Hz, 1H, H-5′), 6.91 (d, J_{trans} = 16.7 Hz, 1H, H-8′), 6.93 (d, J_3 = 7.5 Hz, 1H, H-6′), 7.03 (d, J_{trans} = 16.7 Hz, 1H, H-7′), 7.13 (s, 1H, H-2′), 9.17 (s, 1H, 4′-OH), 12.80 ppm (s, 1H, H–N). ^{13}C NMR ([D_6]DMSO): δ = 55.6 (3′-OCH_3), 99.3 (C-4), 109.5 (C-2′), 112.9 and 118.4 (C-8′), 115.6 (C-5′), 120.1 (C-6′), 128.4 (C-1′),



Scheme 2. The three bioactive pyrazoles.

129.8 (C-7′), 142.0 (C-5), 146.8 (C-4′), 147.9 ppm (C-3′), 151.0 ppm (C-3).

4.1.1.2. (*E*)-3(5)-[β-(4-hydroxy-3-methoxyphenyl)-ethenyl]-5(3)-methyl-1*H*-pyrazole (2). Prepared from β-diketone **9**. The compound was recrystallized from CH_2Cl_2–hexane–EtOH to give a colorless solid (251 mg, 1.1 mmol, 85%). Mp 141.6 °C. Anal. Calcd for $C_{13}H_{14}N_2O_2$ (230.11): C, 67.26; H, 6.44; N, 12.11. Found: C, 67.81; H, 6.13; N, 12.17. ^{1}H NMR ([D$_6$]DMSO): δ = 2.19 (s, 3H, 5(3)-CH_3), 3.81 (s, 3H, 3′-OCH_3), 6.20 (s, 1H, H-4), 6.74 (d, J_3 = 8.1 Hz, 1H, H-5′), 6.88 (d, J_{trans} = 16.7 Hz, 1H, H-8′), 6.91 (br, 1H, H-6′), 6.95 (d, J_{trans} = 16.7 Hz, 1H, H-7′), 7.12 (s, 1H, H-2′), 9.15 (s, 1H, 4′-OH), 12.40 ppm (s, 1H, H–N). ^{13}C NMR ([D$_6$]DMSO): δ = 11.6 (5(3)-CH_3), 55.6 (3′-OCH_3), 101.3 (C-4), 109.5 (C-2′), 115.7 (C-5′), 117.4 (C-8′), 119.9 (C-6′), 128.6 (C-1′), 129.0 (C-7′), 140.5 (C-CH_3), 146.6 (C-4′), 147.9 (C-3′), 149.6 ppm (C–C'_8=C'_7).

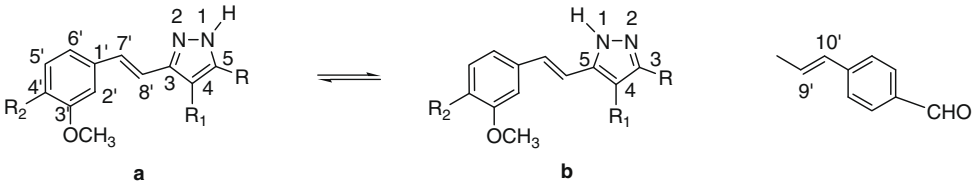
4.1.1.3. (*E*)-3(5)-[β-(4-hydroxy-3-methoxyphenyl)-ethenyl]-4,5(3)-dimethyl-1*H*-pyrazole (3). Prepared from β-diketone **10**. The compound is obtained as a colorless solid after recrystallization from CH_2Cl_2–hexane–EtOH (180 mg, 073 mmol, 61%). Mp 176.1 °C. Anal. Calcd for $C_{14}H_{16}N_2O_2$ (244.12): C, 68.46; H, 6.61; N, 11.35. Found: C, 68.83; H, 6.60; N, 11.47. ^{1}H NMR ([D$_6$]DMSO): δ = 2.03 (s, 3H, 4-CH_3), 2.10 (s, 3H, 5(3)-CH_3), 3.83 (s, 3H, 3′-OCH_3), 6.75 (d, J_3 = 8.2 Hz, 1H, H-5′), 6.86 (d, J_{trans} = 16.6 Hz, 1H, H-8′), 6.91 (dd, J_3 = 8.2 Hz, J_4 = 2.0 Hz, 1H, H-6′), 6.95 (d, J_{trans} = 16.6 Hz, 1H, H-7′), 7.13 (d, J_4 = 1.7 Hz, 1H, H-2′), 9.08 (s, 1H, 4′-OH), 12.29 ppm (s, 1H, H-N). ^{13}C NMR ([D$_6$]DMSO): δ = 8.1 (4-CH_3), 10.6 (5(3)-CH_3), 55.7 (3′-OCH_3), 109.6 (C-2′), 110.4 (C-4), 114.9 (C-8′), 115.6 (C-5′), 119.8 (C-6′), 127.9 (C-7′), 128.8 (C-1′), 141.6 (C-3 or C-5), 146.6 (C-4′), 147.9 ppm (C-3′).

4.1.1.4. (*E*)-3(5)-[β-(4-hydroxy-3-methoxyphenyl)-ethenyl]-5(3)-phenyl-1*H*-pyrazole (4). Prepared from β-diketone **11**. The compound is obtained as a colorless solid after recrystallization from CH_2Cl_2–hexane–EtOH (228 mg, 0.78 mmol, 77%). Mp 142.9 °C. Anal. Calcd for $C_{18}H_{16}N_2O_2$ (292.12): C, 73.95; H, 5.52; N, 10.11. Found: C, 73.95; H, 5.54; N, 9.58. ^{1}H NMR ([D$_6$]DMSO): δ = 3.84 (s, 3H, 3′-OCH_3), 6.78 (d, J_3 = 8.1 Hz, 1H, H-5′), 6.88 (s, 1H, H-4), 6.95 (d, J_{trans} = 16.5 Hz, 1H, H-8′), 6.96 (dd, 1H, J_3 = 8.1 Hz, J_4 = 1.8 Hz, 1H, H-6′), 7.10 (d, J_{trans} = 16.5 Hz, 1H, H-7′), 7.15 (s, 1H, H-2′), 7.31 (t, J_3 = J_3 = 6.7 Hz, 1H, H-*p*), 7.43 (m, 2H, H-*m*), 7.80 (m, 2H, H-*o*), 9.21 (64%) and 9.10 (36%) (s, 1H, 4′-OH), 13.18 (64%) and 12.96 ppm (36%) (s, 1H, H–N). ^{13}C NMR ([D$_6$]DMSO): δ = 55.6 (64%) and 55.5 (36%) (3′-OCH_3), 99.5 (64%) and 100.4 (36%) (C-4), 109.5 (C-2′), 112.7 (64%) and 118.4 (36%) (C-8′), 115.6 (64%) and 115. 3 (36%) (C-5′), 120.2 (64%) and 122.1 (36%) (C-6′), 125.1 (64%) and 125.0 (36%) (C-*o*), 127.5 (C-*p*), 128.1 (C-1′), 128.7 (C-*m*), 130.1 (C-7′), 133.6 (64%) and 132.0 (36%) (C-*ipso*), 142.6 (64%) and 140.3 (36%) (C-5), 147.1 (64%) and 146.6 (36%) (C-4′), 147.9 (C-3′), 151.0 (64%) and 151.4 ppm (36%) (C-3).

4.1.1.5. (*E*)-3(5)-[β-(3,4-dimethoxyphenyl)-ethenyl]-5(3)-phenyl-1*H*-pyrazole (5). Prepared from β-diketone **12**. The compound is obtained as a colorless solid after recrystallization from CH_2Cl_2–hexane–EtOH (196 mg, 1.27 mmol, 51%). Mp 173.4 °C. Anal. Calcd for $C_{19}H_{18}N_2O_2$ (306.14): C, 74.49; H, 5.92; N, 9.14. Found: C, 74.21; H, 5.82; N, 9.16. ^{1}H NMR ([D$_6$]DMSO): δ = 3.78 (s, 3H, 4′-OCH_3), 3.83 (s, 3H, 3′-OCH_3), 6.87 (s, 1H, H-4), 6.96 (d, J_3 = 8.3 Hz, 1H, H-5′), 7.03 (d, J_{trans} = 16.3 Hz, 1H, H-8′), 7.06 (dd, J_3 = 8.3 Hz, J_4 = 1.5 Hz, 1H, H-6′), 7.14 (d, J_{trans} = 16.3 Hz, 1H, H-7′), 7.19 (s, 1H, H-2′), 7.32 (br, 1H, H-*p*), 7.43 (br, 2H, H-*m*), 7.80 (br, 2H, H-*o*), 13.21 (60%) and 13.00 ppm (40%) (s, 1H, H–N). ^{13}C NMR ([D$_6$]DMSO): δ = 55.45 (4′-OCH_3), 55.51 (3′-OCH_3), 99.2 (40%) and 99.8 (60%) (C-4), 108.9 (C-2′), 111.9 (C-5′), 113.6 (C-8′), 119.4 (40%) and 119.9 (60%) (C-6′), 125.0 (C-*o*), 127.4 (C-*p*), 128.6 (C-*m*), 128.9 (40%) and 129.7 (60%) (C-7′), 129.4 (C-1′), 133.7 (C-*ipso*), 142.4 (60%) and 142.8 (40%) (C-5), 149.0 (C-3′), 149.0 (C-4′), 150.9 (60%) and 151.3 ppm (40%) (C-3).

4.1.1.6. (*E*)-3(5)-[β-(3,4-dimethoxyphenyl)-ethenyl]-4-methyl-5(3)-phenyl-1*H*-pyrazole (6). Prepared from β-diketone **13**. The compound is obtained as a colorless solid after recrystallization from CH_2Cl_2–hexane–EtOH (170 mg, 0.53 mmol, 58%). Mp 182.0 °C. Anal. Calcd for $C_{20}H_{20}N_2O_2$ (320.15): C, 74.98; H, 6.29; N, 874. Found: C, 74.28; H, 6.14; N, 8.77. ^{1}H NMR ([D$_6$]DMSO): δ = 2.29 (s, 1H, 4-CH_3), 3.77 (s, 3H, 4′-OCH_3), 3.84 (s, 3H, 3′-OCH_3), 6.95 (d, J_3 = 8.3 Hz, 1H, H-5′), 7.06 (d, J_{trans} = 16.7 Hz, 1H, H-8′), 7.07 (dd, J_3 = 8.3 Hz, J_4 = 1.7 Hz, 1H, H-6′), 7.14 (d, J_{trans} = 16.7 Hz, 1H, H-7′), 7.25 (d, J_4 = 1.7 Hz, 1H, H-2′), 7.34 (br, 1H, H-*p*), 7.45 (br, 2H, H-*m*), 7.65 (br, 2H, H-*o*), 12.94 ppm (s, 1H, H–N). ^{13}C NMR ([D$_6$]DMSO): δ = 9.4 (4-CH_3), 55.5 (4′-OCH_3), 55.6 (3′-OCH_3), 109.1 (C-2′), 110.7 (C-4), 111.9 (C-5′), 114.5 (C-8′), 119.9 (C-6′), 127.1 (C-*o*), 127.3 (C-*p*), 128.4 (C-7′), 128.5 (C-*m*), 130.0 (C-1′), 133.2 (C-*ipso*), 141.7 (C–C'_8=C'_7), 147.1 (C–C_6H_5), 148.8 (C-4′), 149.1 ppm (C-3′).

4.1.1.7. (*E*)-3(5)-[β-4-hydroxy-3-methoxyphenyl)-ethenyl]-5(3)-vinylbenzaldehyde-1*H*-pyrazole (7). Prepared from β-diketone **14**. The resulting product was a yellow solid after recrystallization from CH_2Cl_2–hexane–EtOH (198 mg, 057 mmol, 51%). Mp 228.8 °C.



Scheme 3. NMR atom numbering of pyrazole derivatives.

MS (EI) *m*/*z*: 347 [M+1]. Anal. Calcd for $C_{21}H_{18}N_2O_3$ (346.13): C, 72.82; H, 5.24; N, 8.09. Found: C, 66.41; H, 5.82; N, 11.81. ^{1}H NMR ([D$_6$]DMSO): δ = 3.83 (s, 1H, 3′-OCH$_3$), 6.72 (s, 1H, H-4), 6.78 (d, J_3 = 8.2, 1H, H-5′), 6.93 (d, J_{trans} = 16.2, 1H, H-8′), 6.94 (d, J_3 = 8.2, 1H, H-6′), 7.07 (d, J_{trans} = 16.2, 1H, H-7′), 7.16 (d, J_3 = 9.02, 1H, H-9′), 7.16 (d, J_3 = 9.02, 1H, H-10′), 7.17 (s, 1H, H-2′), 7.62 (m, 2H, H-*o*), 7.67 (64%) and 7.70 (36%) (m, 2H, H-*m*), 7.98 (64%) and 8.13 (36%) (s, 1H, H-CHO), 9.17 (s, 1H, 4′-OH), 11.24 (64%) and 11.36 ppm (36%) (H–N). ^{13}C NMR ([D$_6$]DMSO): δ = 55.6 (3′-OCH$_3$), 100.1 (C-4), 109.5 (C-2′), 112.5 (C-8′), 115.6 (C-5′), 120.1 (C-6′), 126.6 (C-*o*), 127.1 (64%) and 127.4 (36%) (C-*m*), 128.3 (C-1′), 128.3 (C-9′), 128.3 (C-10′), 129.9 (C-7′), 133.5 (C-*p*), 138.1 (60%) and 138.4 (36%) (C-*ipso*), 142.2 (64%) and 145.2 (36%) (CHO), 146.9 (C-4′), 147.9 (C-3′), 150.3 ppm (C-5 or C-3).

4.1.2. General procedure for the preparation of diketones 9–14

Appropriate β-diketone (75 mmol), boric anhydride (3.5 g, 54 mmol) and tri-*n*-butyl borate (23 g, 100 mmol) were mixed in ethyl acetate (50 mL) at 0 °C with stirring. A solution of vanillin (3.75 g, 25 mmol) and *n*-butylamine (0.5 mL) in ethyl acetate was slowly added for 1.5 h. The reaction mixture was stirred at 0 °C for 90 min and then overnight at room temperature. Hydrochloric acid (0.4 N) was then added to the solution, at 60 °C, until pH became acid. The reaction mixture was stirred for an additional hour and then extracted with ethyl acetate. The organic layer was washed with water (10 mL, 2 times), dried (Na_2SO_4) and evaporated under vacuum. The product was isolated by column chromatography with dichloromethane–diethyl ether (95:5) as eluent. The NMR atom numbering for diketones **9**–**14** is reported in Scheme 4.

4.1.2.1. (*E*)-6-(4-Hydroxy-3-methoxyphenyl)hex-5-ene-2,4-dione (feruloylacetone) (9). From 2,4-pentanedione (7.5 g, 75 mmol) to give a yellow solid (2 g, 8.54 mmol, 34%): mp 141–143 °C (lit. 146–147 °C[42]). ^{1}H NMR ($CDCl_3$) δ: 2.16 (s, 3H, CH$_3$-11′), 3.94 (s, 3H, OCH$_3$), 5.63 (s, 1H, CH═COH), 5.83 (s, 1H, OH-4′), 6.32 (d, 1H, *J* = 15.8 Hz, H-8′), 6.92 (d, 1H, *J* = 8.3 Hz, H-5′), 7.02 (d, 1H, J_4 = 1.9 Hz, H-2′), 7.09 (dd, 1H, J_3 = 8.3 Hz, J_4 = 1.9 Hz, H-6′), 7.53 (d, 1H, *J* = 15.8 Hz, H-7′), 15.46 ppm (s, 1H, OH).

4.1.2.2. (*E*)-6-(4-Hydroxy-3-methoxyphenyl)-3-methylhex-5-ene-2,4-dione (10). From 3-methylpentane-2,4-dione (9 g, 75 mmol) to give a yellow solid (800 mg, 3.22 mmol, 13%). Mp 138–140 °C. ^{1}H NMR ($CDCl_3$): δ = 2.01 (s, 3H, CH$_3$-10′), 2.24 (s, 3H, CH$_3$-11′), 3.94 (s, 3H, OCH$_3$-3′), 5.88 (s, 1H, OH-4′), 6.78 (d, 1H, J_{trans} = 15.5 Hz, H-8′), 6.92 (d, 1H, J_3 = 8.2 Hz, H-5′), 7.02 (d, 1H, J_4 = 1.5 Hz, H-2′), 7.13 (dd, 1H, J_3 = 8.2 Hz, J_4 = 1.5 Hz, H-6′), 7.58 (d, 1H, J_{trans} = 15.5 Hz, H-7′), 16.45 ppm (s, 1H, OH).

4.1.2.3. (*E*)-5-(4-Hydroxy-3-methoxyphenyl)-1-phenylpent-4-ene-1,3-dione (11). From 1-phenylbutane-1,3-dione (12 g, 75 mmol) to give a yellow solid (6 g, 20.27 mmol, 82%). Mp 158–161 °C. ^{1}H NMR ($CDCl_3$): δ = 3.95 (s, 3H, OCH$_3$-3′), 5.86 (s, 1H, OH-4′), 6.33 (s, 1H, CH═COH), 6.52 (d, 1H, J_{trans} = 15.8 Hz, H-8′), 6.94 (d, 1H, J_3 = 8.3 Hz, H-5′), 7.07 (d, 1H, J_4 = 1.9 Hz, H-2′), 7.14 (dd, 1H, J_3 = 8.2, J_4 = 1.9 Hz, H-6′), 7.47 (m, 2H, H-*m*), 7.53 (m, 1H, H-*p*), 7.63 (d, 1H, J_{trans} = 15.8 Hz, H-7′), 7.95 (m, 2H, H-*o*), 16.26 ppm (s, 1H, OH).

4.1.2.4. Methylation of (*E*)-5-(4-Hydroxy-3-methoxyphenyl)-1-phenylpent-4-ene-1,3-dione (11). CH$_3$I (190 μl, 2.02 mmol) was added to **11** (600 mg, 2.02 mmol) and K_2CO_3 (280 mg, 2.02 mmol) in dry acetone and the resulting mixture was stirred at room temperature for 48 h. Then, acetone was evaporated under reduced pressure and the residue was purified by column chromatography using chloroform–diethyl ether (95:5) as eluent to give the β-diketones **12** and **13**.

4.1.2.5. (*E*)-5-(3,4-Dimethoxyphenyl)-1-phenylpent-4-ene-1,3-dione (12). The compound is obtained as a yellow solid (100 mg, 032 mmol, 16%). Mp 114–115 °C. ^{1}H NMR ($CDCl_3$): δ = 3.91 (s, 3H, OCH$_3$-4′), 3.93 (s, 3H, OCH$_3$-3′), 6.33 (s, 1H, CH═COH), 6.53 (d, 1H, J_{trans} = 15.8 Hz, H-8′), 6.88 (d, 1H, J_3 = 8.2 Hz, H-5′), 7.09 (d, 1H, J_4 = 1.8 Hz, H-2′), 7.15 (dd, 1H, J_3 = 8.2 Hz, J_4 = 1.8 Hz, H-6′), 7.46 (m, 2H, H-*m*), 7.53 (t, 1H, J_3 = 7.3 Hz, H-*p*), 7.64 (d, 1H, J_{trans} = 15.8 Hz, H-7′), 7.95 (m, 2H, H-*o*), 16.27 ppm (s, 1H, OH).

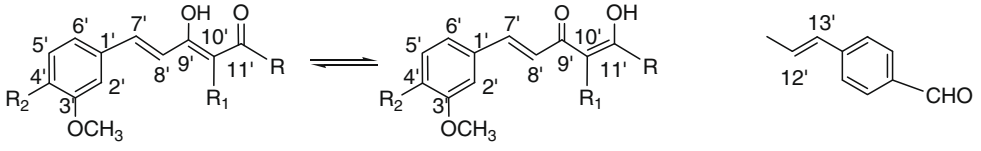
4.1.2.6. (*E*)-5-(3,4-Dimethoxyphenyl)-2-methyl-1-phenylpent-4-ene-1,3-dione (13). The compound is obtained as a yellow pale solid (400 mg, 1.23 mmol, 61%). Mp 112–113 °C. ^{1}H NMR ($CDCl_3$): δ = 1.54 (s, 3H, CH$_3$-10′), 3.89 (s, 6H, OCH$_3$-3′ and OCH$_3$-4′), 6.69 (d, 1H, J_{trans} = 15.8 Hz, H-8′), 6.83 (d, 1H, J_3 = 8.3 Hz, H-5′), 7.01 (dd, 1H, J_4 = 1.4 Hz, H-2′), 7.10 (dd, 1H, J_3 = 8.3 Hz, J_4 = 1.4 Hz, H-6′), 7.45 (m, 2H, H-*m*), 7.55 (t, 1H, J_3 = 7.6 Hz, H-*p*), 7.60 (d, 1H, J_{trans} = 15.8 Hz, H-7′), 8.00 ppm (m, 2H, H-*o*).

4.1.2.7. 4-((1*E*,6*E*)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dienyl) benzaldehyde (14). Feruloylacetone **9** (500 mg, 2.14 mmol), boric anhydride (230 mg, 3.20 mmol) and tri-*n*-butyl borate (2.5 g, 10.05 mmol) in DMF (10 ml) were heated at 80 °C, for 1 h. A solution of terephthaldicarboxaldehyde (145 mg, 1.07 mmol) and *n*-butylamine (0.15 ml) in DMF (2 ml) was slowly added (60 min). After 5 h, the reaction mixture was cooled down to 60 °C and hydrochloric acid (0.4 N) was added until the reaction mixture reached pH 2–3. The mixture was stirred for 1 h and extracted with dichloromethane. The organic layer was washed with water (10 mL, 2 times), dried (Na_2SO_4) and evaporated under vacuum. The solid residue was purified by column chromatography (eluent ethyl acetate–hexane 1:1giving compound **14** as an orange solid (194 mg, 0.55 mmol, 52%). Mp 209–211 °C. MS (EI) *m*/*z*: 351 [M+1]. ^{1}H NMR ([D$_6$]DMSO): δ = 3.84 (s, 3H, 3′-OCH$_3$), 6.18 (s, 1H, H-10′), 6.82 (d, 1H, J_{trans} = 16.0 Hz, H-8″), 6.83 (d, 1H, J_3 = 8.1 Hz, H-5′), 7.09 (d, 1H, J_{trans} = 15.9 Hz, H-9′), 7.18 (dd, 1H, J_3 = 8.1 Hz, J_4 = 1.7 Hz, H-6′), 7.35 (d, 1H, J_4 = 1.3 Hz, H-2′), 7.62 (d, 1H, J_{trans} = 16.0 Hz, H-7′), 7.93 (m, 2H, H-*o*), 7.96 (m, 2H, H-*m*), 9.71 (s, 1H, 4′-OH), 10.03 (s, 1H, CHO), 16.14 ppm (s, 1H, OH).

4.2. Biological methods

4.2.1. Cell culture

Caco-2 cells, human intestinal epithelial cell line, were obtained from European Collection of Cell Cultures (ECACC). Cells were cultured in 75-cm^2 flasks in a humidified atmosphere containing 5% CO_2 at 37 °C.



Scheme 4. NMR atom numbering of diketone derivatives in the enol forms.

For the in vitro experiments, intestinal cells were seeded into 24-well plates in free serum-medium and cultured in the presence or absence of the combination of human recombinant TNF-α (1, 10 and 100 ng/mL) and IL-1β (1, 10 and 10 ng/mL) for 24 h. In addition, cells were incubated in the presence of different synthetic curcumin pyrazole derivatives at different concentrations (1, 5, 10, 25, 50 μM) 1 h before adding cytokines. Afterwards, conditioned medium was collected for the study of MMP activity by zymography assay. Further experiments were performed with curcumin at the same doses.

4.2.2. Zymography

Gelatine zymography was used to detect MMP-9 and MMP-2 activity. Zymographic analysis was carried out on conditioned media as previously described (12). Briefly, zymography was performed subjecting samples to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with copolymerized gelatin (0.2%; Sigma Chemical Co, St. Louis, Mo) incorporated as a substrate for gelatinolytic proteases. After electrophoresis, the gels were washed with 2% Triton X-100 (3 times, 20 minutes each), and then incubated in development buffer (50 mml Tris HCl, 200 mmol NaCl, 10 mmol $CaCl_2$ and 1 μmol $ZnCl_2$, pH 7.5) at 37C overnight. Internal standard, conditioned medium of HT-1080 human fibrosarcoma cells (that contains high amounts of proMMP-2, MMP-2, proMMP-9 and MMP-9), were run as controls on the gels. After 48 h development, gels were fixed and stained in 40% methanol, 10% acetic acid and 0,1% (wt/v) Coomassie Blue R-250 (Sigma Chemical Co, St. Louis, Mo) for 1 h and then de-stained. Relative molecular weights of clear bands were analyzed in comparison to the standards using a calibrated densitometer (GS-800, BioRad) and QuantityOne Quantitation analysis software (BioRad, version 4). Zymographic activity of each band was measured in terms of Optical Density (OD)/mm2.

4.2.3. Western-blotting

Western-blot analysis of MMP-9 was performed in Caco-2 conditioned media, as previously described.[41] Samples were denatured and loaded (50 μg/lane) onto 10% (vol/vol) polyacrylamide gels. Following electrophoresis, proteins were transferred onto nitrocellulose membrane (Protran, Schleider&Schuell) and detected using a rabbit monoclonal anti-MMP-9 antibody (Chemicon, Ca, USA) at 1:1000 concentration and a chemiluminescent substrate (Pierce, Rockford, USA). To account for the inter-blot variations in MMP immunoreactivity, internal standard (human recombinant MMP-9) was used. Relative molecular weights of clear bands were analyzed in comparison to the standard using a calibrated densitometer (GS-800, BioRad) and QuantityOne Quantitation analysis software (BioRad, version 4). Protein expression of each band was measured in terms of Optical Density (OD)/mm2.

Acknowledgements

This work has been financed by the Spanish MEC (CTQ2006-02586 and CTQ2007-62113) and by Stokes Science Foundation of Ireland (SFI) program. Thanks are given to Dr. Sergio Erill and Dr. Sebastian Videla that promoted the collaborative research.

References and notes

1. Fiocchi, C. *Am. J. Physiol. Gastrointest. Liver Physiol.* **1997**, *273*, G769.
2. Podolsky, D. K. *N. Engl. J. Med.* **2002**, *34*, 417.
3. Baugh, M. D.; Perry, M. J.; Hollander, A. P.; Davies, D. R.; Cross, S. S.; Lobo, A. J.; Taylor, C. J.; Evans, G. S. *Gastroenterology* **1999**, *117*, 814.
4. Heuschkel, R. B.; MacDonald, T. T.; Monteleone, G.; Bajaj-Elliott, M.; Smith, J. A. W.; Pender, S. L. *Gut* **2000**, *47*, 57.
5. Kirkegaard, T.; Hansen, A.; Bruun, E.; Brynskov, J. *Gut* **2004**, *53*, 701.
6. Medina, C.; Radomski, M. W. *J. Pharmacol. Exp. Ther.* **2006**, *318*, 933.
7. Von Lampe, B.; Barthel, B.; Coupland, S. E.; Riecken, E. O.; Rosewicz, S. *Gut* **2000**, *47*, 63.
8. Di Sebastiano, P.; di Mola, F. F.; Artese, L.; Rossi, C.; Mascetta, G.; Pernthaler, H.; Innocenti, P. *Digestion* **2001**, *63*, 234.
9. Medina, C.; Santana, A.; Llopis, M.; Paz-Cabrera, M. C.; Antolin, M.; Mourelle, M.; Guarner, F.; Vilaseca, J.; Gonzalez, C.; Salas, A.; Quintero, E.; Malagelada, J.-R. *Inflamm. Bowel Dis.* **2005**, *11*, 99.
10. Medina, C.; Santana, A.; Paz, M. C.; Diaz, F.; Farre, E.; Salas, A.; Radomski, M. W.; Quintero, E. *J. Leukocyte Biol.* **2006**, *79*, 954.
11. Medina, C.; Videla, S.; Radomski, A.; Radomski, M.; Antolin, M.; Guarner, F.; Vilaseca, J.; Salas, A.; Malagelada, J.-R. *Scand. J. Gastroenterol.* **2001**, *36*, 1314.
12. Medina, C.; Videla, S.; Radomski, A.; Radomski, M. W.; Antolin, M.; Guarner, F.; Vilaseca, J.; Salas, A.; Malagelada, J.-R. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2003**, *284*, G116.
13. Jovanovic, S. V.; Steenken, S.; Boone, C. W.; Simic, M. G. *J. Am. Chem. Soc.* **1999**, *121*, 9677.
14. Masuda, T.; Hidaka, K.; Shinohara, A.; Maekawa, T.; Takeda, Y.; Yamaguchi, H. *J. Agric. Food Chem.* **1999**, *47*, 71.
15. Weber, W. M.; Hunsaker, L. A.; Abcouwer, S. F.; Deck, L. M.; Vander Jagt, D. L. *Bioorg. Med. Chem.* **2005**, *13*, 3811.
16. Brouet, I.; Ohshima, H. *Biochem. Biophys. Res. Commun.* **1995**, *206*, 533.
17. Arbiser, J. L.; Klauber, N.; Rohan, R.; van Leeuwen, R.; Huang, M. T.; Fisher, C.; Flynn, E.; Byers, H. *Mol. Med.* **1998**, *4*, 376.
18. Jacob, A.; Wu, R.; Zhou, M.; Wang, P. *PPAR Res.* **2007**, 89369.
19. Sharma, R. A.; Ireson, C. R.; Verschoyle, R. D.; Hill, K. A.; Williams, M. L.; Leuratti, C.; Manson, M. M.; Marnett, L. J.; Steward, W. P.; Gescher, A. *Clin. Cancer Res.* **2001**, *7*, 1452.
20. Füllbeck, M.; Huang, X.; Dumdey, R.; Frommel, C.; Dubiel, W.; Preissner, R. *BMC Cancer* **2005**, *5*, 97.
21. Siwak, D. R.; Shishodia, S.; Aggarwal, B. B.; Kurzrock, R. *Cancer* **2005**, *104*, 879.
22. Egan, M. E.; Pearson, M.; Weiner, S. A.; Rajendran, V.; Rubin, D.; Glockner-Pagel, J.; Canny, S.; Du, K.; Lukacs, G. L.; Caplan, M. J. *Science* **2004**, *304*, 600.
23. Sundaryono, A.; Nourmamode, A.; Gardrat, C.; Fritsch, A.; Castellan, A. *J. Mol. Struct.* **2003**, *649*, 177.
24. Zsila, F.; Bikadi, Z.; Simonyi, M. *Org. Biomol. Chem.* **2004**, *2*, 2902.
25. Kong, L.; Priyadarsini, I.; Zhang, H.-Y. *J. Mol. Struct. (TheoChem)* **2004**, *684*, 111.
26. Kim, G.-Y.; Kim, K.-H.; Lee, S.-H.; Yoon, M.-S.; Lee, H.-J.; Moon, D.-O.; Lee, C.-M.; Ahn, S.-C.; Park, Y. C.; Park, Y.-M. *J. Immunol.* **2005**, *174*, 8116.
27. Aggarwal, B. B.; Kumar, A.; Aggarwal, M. S.; Shishodia, S. In *Phytochemicals in Cancer Chemoprevention*; Bagchi, D., Preuss, H. G., Eds.; CRC Press: Boca Raton, 2005; p 349.
28. Maheshwari, R. K.; Singh, A. K.; Gaddipati, J.; Srimal, R. C. *Life Sci.* **2006**, *78*, 2081.
29. *The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease*; Aggarwal, B. B., Shishodia, S., Surh, Y.-J., Eds.; Springer Publishers: New York, 2007.
30. Wright, J. S. *J. Mol. Struct. (TheoChem)* **2002**, *591*, 207.
31. Jayaprakasha, G. K.; Jaganmohan Rao, L.; Sakariah, K. K. *Food Chem.* **2006**, *98*, 720.
32. Flynn, D. L.; Belliotti, T. R.; Boctor, A. M.; Connor, D. T.; Kostlan, C. R.; Nies, D. E.; Ortwine, D. F.; Schrier, D. J.; Sircar, J. C. *J. Med. Chem.* **1991**, *34*, 518.
33. Shim, J. S.; Kim, D. H.; Jung, H. J.; Kim, J. H.; Lim, D.; Lee, S.-K.; Kim, K.-W.; Ahn, J. W.; Yoo, J.-S.; Rho, J.-R.; Shin, J.; Kwon, H. J. *Bioorg. Med. Chem.* **2002**, *10*, 2439.
34. Ishida, J.; Ohtsu, H.; Tachibana, Y.; Nakanishi, Y.; Bastow, K. F.; Nagai, M.; Wang, H.-K.; Itokawa, H.; Lee, K.-H. *Bioorg. Med. Chem.* **2002**, *10*, 3481.
35. Ohtsu, H.; Xiao, Z.; Ishida, J.; Nagai, M.; Wang, H. K.; Itokawa, H.; Su, C. Y.; Shih, C.; Chiang, T.; Chang, E.; Lee, Y. F.; Tsai, M. Y.; Chang, C.; Lee, K. H. *J. Med. Chem.* **2002**, *45*, 5037.
36. Selvam, C.; Jachak, S. M.; Thilagavathi, R.; Chakraborti, A. K. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1793.
37. Narlawar, R.; Baumann, K.; Schubenel, R. *Neurodegener Dis.* **2007**, *4*, 88.
38. Narlawar, R.; Pickhardt, M.; Leuchtenberger, S.; Baumann, K.; Krause, S.; Dyrks, T.; Weggen, S.; Mandelkow, E.; Schmidt, E. *ChemMedChem* **2008**, *3*, 65.
39. Levin, J. I.; Gu, Y.; Nelson, F. C.; Zask, A.; DiJoseph, J. F.; Sharr, M. A.; Sung, A.; Jin, G.; Cowling, R.; Chanda, P.; Cosmi, S.; Hsiao, C.-L.; Edris, W.; Wilhelm, J.; Killar, L. M.; Skotnicki, J. S. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 239.
40. Shishodia, S.; Koul, D.; Aggarwal, B. B. *J. Immunol.* **2004**, 2011.
41. Donnini, S.; Monti, M.; Castagnini, C.; Solito, R.; Botta, M.; Schenone, S.; Giachetti, A.; Ziche, M. *Int. J. Cancer* **2007**, *120*, 995.
42. Cornago, M. P.; Cabildo, M. P.; Claramunt, R. M.; Bouissane, L.; Pinilla, E.; Torres, M. R.; Elguero, J. *New J. Chem.* **2009**, *33*, 125.
43. Castaneda, F. E.; Walia, B.; Vijay-Kumar, M.; Patel, N. R.; Roser, S.; Kolachala, V. L.; Rojas, M.; Wang, L.; Oprea, G.; Garg, P.; Gewirtz, A. T.; Roman, J.; Merlin, D.; Sitaraman, S. V. *Gastroenterology* **2005**, *129*, 1991.
44. Santana, A.; Medina, C.; Paz-Cabrera, M.; Díaz-Gonzalez, F.; Farre, E.; Salas, A.; Radomski, M. W.; Quintero, E. *World J. Gastroenterol.* **2006**, *12*, 6464.
45. Gan, X.; Wong, B.; Wright, S. D.; Cai, T.-Q. *J. Interferon Cytokine Res.* **2001**, *21*, 93.
46. Huhtala, P.; Tuuttila, A.; Chow, L. T.; Lohi, J.; Keski-Oja, J.; Tryggvason, K. *J. Biol. Chem.* **1991**, *266*, 16485.
47. Huhtala, P.; Eddy, R. L.; Fan, Y. S.; Byers, M. G.; Shows, T. B.; Tryggvason, K. *Genomics* **1990**, *6*, 554.
48. Shakibaei, M.; John, T.; Schulze-Tanzil, G.; Lehmann, I.; Mobasheri, A. *Biochem. Pharmacol.* **2007**, *73*, 1434.
49. Saja, K.; Babu, M. S.; Karunagaran, D.; Sudhakaran, P. R. *Int. Immunopharmacol.* **2007**, *7*, 1659.
50. Berger, S.; Braun, S. *In 200 More NMR Experiments: A Practical Course*; J Wiley & Sons: New York, 2004.
51. Payton, F.; Sandusky, P.; Alworth, W. L. *J. Nat. Prod.* **2007**, *70*, 143.