Cell. Mol. Life Sci. 62 (2005) 2896–2903
1420-682X/05/232896-8
DOI 10.1007/s00018-005-5422-7

Cellular and Molecular Life Sciences

Research Article

A structure-activity study for the inhibition of metalloproteinase-9 activity and gene expression by analogues of gallocatechin-3-gallate

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Received 14 September 2005; received after revision 6 October 2005; accepted 12 October 2005
Online First 28 November 2005

Abstract. Catechins are able to modulate the gelatinolytic activity of matrix metalloproteinase-9 (MMP-9) by reducing its release from macrophages. Gallocatechins decrease MMP-9 secretion by lowering MMP-9 promoter activity and mRNA levels. The effect appears to be dependent on some structural and stereochemical requirements. In this study, the relationship between chemical structure and activity was studied by testing the effect of analogues of (±)-gallocatechin-3-gallate (±)-GCG, selectively deprived of hydroxyl groups, on MMP-9 activity, transcription, and secretion. Our results indicate that (±)-GCG and (±)-catechin-3-gallate are characterized by a substitution pattern compatible with direct inhibition of MMP-9 activity. Conversely, when transcription was the target, (±)-*trans*-3-flavanol-3-benzoate, lacking all the hydroxyl groups, was the most effective both in lowering MMP-9 promoter activity and consequently protein secretion, and in inhibiting nuclear-factor-κB-driven transcription. Our results suggest that the structural requirements for enzyme inhibition are different from those necessary for targeting gene expression.

Key words. Gelatinase B; matrix metalloproteinase-9; gene expression; structure-activity relationship; catechins; gallocatechin-3-gallate; NF-κB driven-transcription

Matrix metalloproteinases (MMPs) are a family of structurally related, zinc-containing enzymes, and more than 20 different MMPs have been described [1]. MMPs are secreted as proenzymes and their activity is regulated by a pro-domain that maintains the proteinase in a latent zymogen state through a cysteine switch mechanism [2]. Once activated, MMPs play an important role in the remodeling of the extracellular matrix (ECM) under physiological situations, such as fetal tissue development and postnatal tissue repair. Conversely, overexpression of these proteinases leads to an excessive breakdown of ECM that occurs in many pathological conditions like cancer invasion and metastasis [3], cartilage destruction in arthritis, atherosclerotic plaque rupture, and the development of aneurysms [4]. Among the several families of MMPs, the 92-kDa gelatinase B (MMP-9) is expressed by virtually all activated macrophages, facilitating, through the degradation of the basement membrane, macrophage extravasation. MMP-9 is present in atherectomy materials from unstable angina [5] and abdominal aortic aneurysm [6]. In addition, several studies revealed that the suppression of MMP-9 reduces the invasive and metastatic ability of tumor cells [3].

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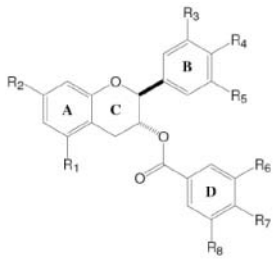
Green tea polyphenols have been reported to suppress gelatin degradation mediated by MMP-2 and MMP-9 under several conditions [for a recent review see ref. 7]. A green tea extract rich in polyphenols inhibited MMP-9 activity up to 80% at 35 μg/ml [8] and this effect was correlated with the presence of (–)-epicatechin-3-gallate [(–)-(ECG)] and (–)-epigallocatechin-3-gallate [(–)-EGCG)], the most prevalent flavanols in green tea. (–)-EGCG was also shown to inhibit the secretion of gelatinases from the highly metastatic human fibrosarcoma cell line HT1080 [9]. The mechanism or mechanisms that enable catechins to down-regulate MMP-9 secretion have been extensively investigated: recent studies demonstrated that (–)-EGCG-mediated suppression of MMP-9 secretion was correlated with decreased levels of MMP-9 mRNA, consequent to the inhibition of ERK 1/2, members of a MAPK family necessary for MMP-9 up-regulation [9]. Similarly, (–)-EGCG blocked tumor-promoter-induced MMP-9 expression via the suppression of MAPK and the activation of AP-1 in human gastric cells [10]. Several studies demonstrated the ability of (–)-EGCG to interfere with the NF-κB pathway, thus reducing MMP-9 expression during inflammation and proliferation [for a review, see ref. 7]. Through structure-activity relationship (SAR) studies, both the galloyl group and the planarity of the molecules appear to be important elements in conferring an inhibitory activity against gelatinases [11] and other enzymes, such as fatty acid synthase [12] and squalene epoxidase [13]. On the other hand, the stereochemistry of the C ring seems not to be relevant for the enzymatic interaction, since both (+)-EGCG and (–)-EGCG were equally active on proteasome, a multicatalytic protease responsible for the degradation of most cellular proteins, probably due to the partial symmetry of their A-C rings [14]. Results obtained in our laboratory [15] showed that only gallocatechin isomers with C2 (R) configuration were able to down-regulate MMP-9 promoter activity. In agreement with our findings, the antitumor-promoting activity of catechins is stereochemically dependent [16]. Other SAR studies with catechins have focused on the importance of hydroxyl groups (either the number and the position on the flavan skeleton) in modulating their antioxidant and anti-scavenger activities [17, 18], or their anti proliferative activity in human stomach cancer cells [19].

The role of hydroxyl groups (number and position) of catechins in determining the effects on MMP-9 activity and transcription has not yet been investigated. Thus, in the present study, analogues of (±)-GCG were synthesized to selectively deprive the flavan skeleton of phenolic hydroxyl groups (fig. 1). In general, catechins are obtained from natural sources as a mixture of (+) and/or (–) isomers. The total synthesis of (–)-EGCG and of an ester analogue have been recently reported [20, 21]. According to these methodologies, the (±)-*trans* stereoisomer of gallocatechins is predominantly obtained and further synthetic steps are necessary to synthesize the cis stereoisomer (corresponding to (–)-EGCG). Therefore, for the purpose of our study, only the (±)-*trans* isomers were prepared because of the easier synthetic accessibil-

Figure 1. Structures of catechin analogues.



	name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	(±)-gallocatechin-3-gallate	OH	OH	OH	OH	OH	OH	OH	OH
2	(±)-afzelechin-3-gallate	OH	OH	H	OH	H	OH	OH	OH
3	(±)-gallocatechin-3-(p-salicylate)	OH	OH	OH	OH	OH	H	OH	H
4	(±)-robidanol-3-gallate	H	OH	OH	OH	OH	OH	OH	OH
5	(±)-*trans*-3,3',4',5'-flavantetrol-3-gallate	H	H	OH	OH	OH	OH	OH	OH
6	(±)-catechin-3-gallate	OH	OH	H	OH	OH	OH	OH	OH
7	(±)-catechin-3-(α-resorcylate)	OH	OH	H	OH	OH	OH	H	OH
8	(±)-*trans*-3-flavanol-3-benzoate	H	H	H	H	H	H	H	H
9	(±)-oxykoaburagenin-3-gallate	OH	OH	H	H	H	OH	OH	OH
10	(±)-gallocatechin-3-benzoate	OH	OH	OH	OH	OH	H	H	H

ity. The synthesized compounds were tested for their effects on MMP-9 catalytic activity, transcription, and secretion. Since MMP-9 gene expression may be regulated by NF-κB, the effect of the synthesized analogues on NF- κB driven transcription was also investigated.

Materials and Methods

Chemicals. All reagents and solvents for the synthesis were purchased from commercial suppliers (Sigma-Aldrich-Fluka-RiedeldeHaën, Milan, Italy; Acros Organics, Geel, Belgium; Lancaster Synthesis, Lancaster, UK; Iris Biotech, Marktredwitz, Germany) and used without further purifications. All other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany).

Cell culture reagents were purchased from Invitrogen (Life Technologies, Milan, Italy). The plasmid 2.2-Luc, containing the promoter fused to a luciferase reporter gene [22], was a kind gift of C. K. Glass (Department of Cellular and Molecular Medicine, School of Medicine, University of California, San Diego, CA, USA). A luciferase reporter plasmid with three κB sites from the E-selectin promoter was previously described [23] and kindly provided by N. Marx (Department of Internal Medicine II-Cardiology, University of Ulm, Ulm Germany). The pCMVβ-galactosidase plasmid was from Clontech (Palo Alto, CA, USA). Restriction enzymes and luciferin were obtained from Promega (Milan, Italy). The plasmid purification kit was purchased from Qiagen (Milan, Italy). The African green monkey cell line CV-1 and human hepatoma HepG2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). C57BL/6J female mice were purchased from Charles River (Calco, Italy). Dulbecco's modified eagle's medium (DMEM) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Milan, Italy).

Synthesis of catechin analogues. Compounds **1–10** were prepared as outlined in figure 2, and their structure is given in figure l, thus the aldolic condensation between

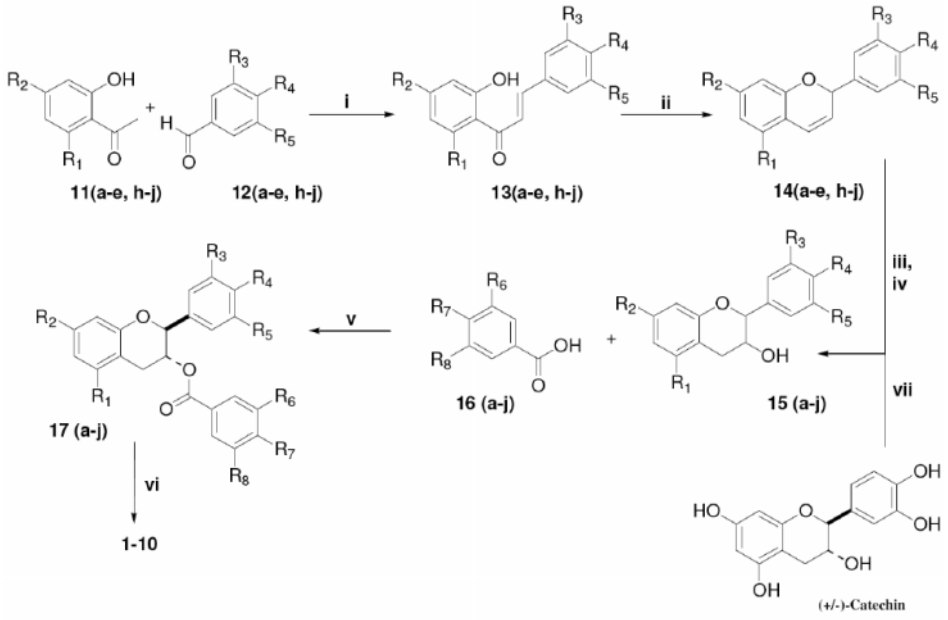


Figure 2. Reagents and conditions: (i) NaH, N,N-dimethylformamides (DMF), 0 °C; (ii) $NaBH_4$, tetrahydrofuran (THF)/EtOH; (iii) BH_3-THF; (iv) NaOH, H_2O_2; (v) O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate, (HBTU), 4-dimethylaminopyridine (DMAP), 4-methylmorpholine (NMM), DMF; (vi) H_2, palladium on carbon, THF/MeOH; (vii) BnBr, NaH, DMF.

11-17	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
a	OBn	OBn	OBn	OBn	OBn	OBn	OBn	OBn
b	OBn	OBn	H	OBn	H	OBn	OBn	OBn
c	OBn	OBn	OBn	OBn	OBn	H	OBn	H
d	H	OBn	OBn	OBn	OBn	OBn	OBn	OBn
e	H	H	OBn	OBn	OBn	OBn	OBn	OBn
f	OBn	OBn	H	OBn	OBn	OBn	OBn	OBn
g	OBn	OBn	H	OBn	OBn	OBn	H	OBn
h	H	H	H	H	H	H	H	H
i	OBn	OBn	H	H	H	OBn	OBn	OBn
j	OBn	OBn	OBn	OBn	OBn	H	H	H

o-hydroxyacetophenones **11** and benzaldehydes **12** (step i) gave calchones **13** [24]. 3-Flavenes **14** were obtained by reductive cyclization of calchones with $NaBH_4$ (step ii) [20]; the conversion of compound **14** into a trialkylborane complex (step iii), and the subsequent cleavage by hydrogen peroxide gave 3-flavanols **15** (step iv), isolated as a mixture of *trans* and *cis* diastereoisomers (*trans/cis* = 4/1). Coupling of flavanols **15** with benzoic acids **16** and isolation of the trans isomers afforded esters **17** (step v) which were then deprotected by catalytic hydrogenation (step vi), giving final products **1–10**. Flavanols **15f** and **15g** were prepared from commercially available (±)-catechin (step vii) by selective benzyl protection of phenolic hydroxyl groups [25].

Cell culture. Mouse peritoneal macrophages were collected by peritoneal lavage with phosphate-buffered saline (PBS) from C57BL/6J mice and plated as described previously [26].

CV-1 and HepG2 cells were plated in 24-well plates (10^5 cells/well) the day before transfection in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum.

To generate the conditioned media, macrophages were incubated for 24 h at 37 °C with DMEM/F-12 nutrient mixture (DMEM/F-12) supplemented with 0.2% BSA in the absence or presence of the compounds under study (10 μM). At the end of the incubation, the conditioned media were collected and the gelatinolytic capacity of secreted MMP-9 was evaluated by zymography. Cellular protein content was measured according to Lowry et al. [27]. For quantitation of zymograms, densitometric scanning was performed using a system incorporating a video camera and a computer analysis package (NIH Image 1.52 image analysis software). Each experiment was performed at least twice with different preparations of cells. Results were normalized by cellular protein content and expressed as arbitrary optical density (O.D.) units. Data are presented as the mean ± S.D. and analyzed using the Dunnett test.

SDS-PAGE zymography. MMP-9 gelatinolytic activity was evaluated as previously described [28]. Briefly, samples underwent electrophoresis on 7.5% polyacrylamide gels containing 10% SDS and gelatin (1 mg/ml). The gels were then washed in 2.5% Triton X-100 (Sigma) at room temperature and then incubated overnight at 37 °C (Tris 50 mM pH 7.5 containing NaCl 150 mM, $CaCl_2$ 10 mM, $ZnCl_2$ 1 μM; activation buffer). At the end of the incubation, the gels were stained with Coomassie brilliant blue R-250 (Sigma-Aldrich). To test the direct effect of synthesized compounds **1–10** on the activity of secreted MMP-9, conditioned media obtained from untreated cells were electrophoresed as described above and the tested compounds added in the activation buffer.

Transient transfection assay. Transfections of CV-1 and HepG2 cells were performed by the calcium phosphate co-precipitation technique [29]. Briefly, a unique co-precipitate containing each reporter plasmid/luciferase plus pCMVβ-gal was prepared and aliquoted in different wells to ensure that all samples were transfected with the same amount of plasmid DNA (1.2 μg of luciferase plasmid plus 0.3 μg of β-galactosidase plasmid DNA/well). After 16 h at 37 °C, CV-1 cells were washed with PBS and incubated for 24 h in medium containing the compounds (0.5–20 μM) to be tested or the vehicle (ethanol or DMSO, 0.1%) in the presence of phorbol myristate acetate 100 nM. Four hours post-transfection, HepG2 cells were incubated in medium without fetal calf serum in the absence or presence of the compounds (10–30 μM).

Enzyme assay. Luciferase and β-galactosidase assays were performed using a luminometer (Lumat 9501, Berthold, Germany) and a microtiter plate reader (Bio-Rad, Hercules, Calif.) respectively, as previously described [29]. Luciferase activities were normalized versus galactosidase activities. Results are expressed as the inhibition of normalized luciferase activities versus control and represent the mean ± SD values of triplicate samples. Each experiment was repeated at least twice. Statistical analyses were performed using the Student's t test.

Cytotoxicity assay. Cellular toxicity caused by the compounds was assessed both by measuring cellular protein [27] and by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) colorimetric assay [30]. CV-1 and HepG2 cells were treated with compounds (up to 20 μM) for 24 h in DMEM supplemented with 10% heat-inactivated FBS. The medium was removed, and cells were incubated with a solution containing MTT 0.5 mg/ml in PBS at 37 °C for 3 h. The MTT solution was removed and MTT formazan was extracted with isopropanol:DMSO (9:1, v/v; 500 μl/well) for 15 min at 37 °C. Aliquots of 100 μl were read on a plate reader (Bio-Rad Laboratories) at 560 nm (reference wavelength 690 nm). No sign of cytotoxicity was seen with the concentrations used for the experiments.

Results

Effect of catechins on MMP-9 activity. In the first set of experiments, the ability of catechins **1–10** to affect directly the *in vitro* gelatinolytic activity of MMP-9 was investigated. In these experiments, (–)-EGCG was used as reference compound. As shown in figure 3, at 10 μM, only compounds **1** (–80%), **6** (–70%), **2** and **10** (–20%) inhibited MMP-9 activity, with compound **1** being even more active than the reference compound (–)-EGCG. The effect of the other catechins was negligible.

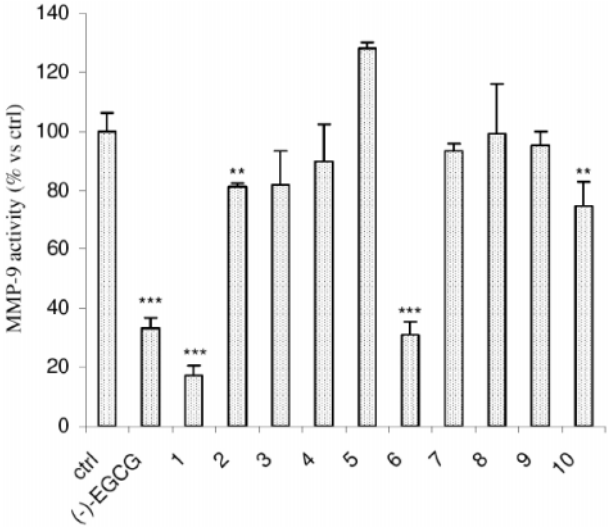


Figure 3. Effect of catechin analogues (10 μM) on the activity of secreted MMP-9. (–)-EGCG (30 μM) was used as reference compound. Data were quantified by densitometry scanning and expressed as the mean ± SD of two experiments performed in duplicate. ** $p < 0.01$; *** $p < 0.001$ versus control.

Table 1. Effect of catechin analogues (10 μM) on MMP-9 secretion and promoter activity.

Percent inhibition versus control (mean ± SD)		
Compound	MMP-9 secretion	MMP-9 promoter activity
(–)-EGCG	47.6 ± 9.9 **	29.0 ± 9.4 *
1	45.7 ± 10.5 **	32.4 ± 12.9 *
2	N.S.	N.S.
3	N.S.	40.6 ± 6.6 **
4	31.6 ± 3.4 **	47.7 ± 14.2 **
5	N.S.	37.8 ± 5.1 **
6	N.S.	N.S.
7	20.5 ± 13.5 *	26.7 ± 8.7 *
8	68.0 ± 1.1 ***	62.0 ± 13.7 ***
9	N.S.	N.S.
10	12.3 ± 1.0**	11.2 ± 5.6*

Data on MMP-9 secretion were quantified by densitometry scanning and expressed as the mean ± SD of three experiments performed in duplicate. Results on MMP-9 promoter activity were expressed as the inhibition of normalized luciferase activities versus control and represent the mean ± SD of three experiments performed in triplicate. N.S., not statistically significant vs controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control.

Effect of catechins on MMP-9 secretion and transcription. We then evaluated the effect of the compounds on MMP-9 secretion by macrophages. As shown in table 1, compounds **1**, **4**, **7**, **8** and **10** (at 10 μM) reduced MMP-9 secretion by 10–70%, with compound **8** being the most effective.

To verify whether the down-regulation of MMP-9 secretion was consequent to a decreased rate of MMP-9 gene transcription, we tested the effect of the synthesized catechins on MMP-9 promoter activity. CV-1 cells were used because they are easily transfectable, unlike macrophage cell lines, and secrete measurable amounts of MMP-9, as assessed by gelatin zymography (data not shown). As shown in table 1, compound **8** was the most active (62.0% inhibition); compounds **1**, **3, 4**, **5** and **7,** showed a comparable effect (inhibition in the range of 27–48%, difference not statistically significant) while compound **10** showed only a negligible effect (10% inhibition). For comparison, inhibition by (–)-EGCG was 29%. Compounds **2**, **6** and **9** characterized by the removal of one, two or three hydroxyls group from the B ring system, were inactive.

From concentration-inhibition curves performed with **8**, **4** and **1**, the concentrations required to obtain 50% inhibition were calculated. As shown in figure 4, compound **8** was the most potent, with an IC_{50} of 4.17 ± 0.9 μM, whereas for **1** and **4**, 50% inhibition was obtained at 20 μM. The inhibition by (–)-EGCG was 55% at 20 μM, as reported elsewhere [9]. Concentration-dependent curves of the inhibitory effect for **8** and **4** were statistically different with respect to **1** ($p = 0.0002$ for **8** vs **1** and $p = 0.004$ for **4** vs **1**).

The inhibitory effect on MMP-9 promoter-induced transcription by compound **8** was not due to a generalized suppression of gene transcription since the compound did not affect SV40 early gene promoter-driven transcription (data not shown).

The data in table 1 show that the extent of the inhibition of MMP-9 secretion by compounds **8**, **4**, **1**, **7** and **10** mirrored that observed for promoter activity, and the two effects were correlated ($r = 0.87$); compounds inactive on the promoter activity did not affect MMP-9 secretion either. Only for compounds **5** and **3** we could not see a correlation between the effect on the promoter and the enzyme secretion.

Effect of catechins on NF-κB-driven-transcription. To determine whether the inhibition of MMP-9 gene expression by compounds **8**, **4** and **1** could be ascribed to a reduction in activity of the NF-κB pathway, we measured the effect of these compounds (at 10–30 μM) on NF-κB-driven transcription. As shown in figure 5, the inhibitory effect on NF-κB exerted by the compounds followed the same order as for the inhibition of MMP-9 promoter activity: **8 > 4 > 1** (72.1%, 53.6%, and 31%, respectively, at 30 μM). The inhibition by **1** was similar to that exerted by (–)-EGCG, the reference compound (35.2% at 30 μM). There is a direct correlation ($r = 0.79$) between the effect on MMP-9 promoter activity and on NF-κB-driven

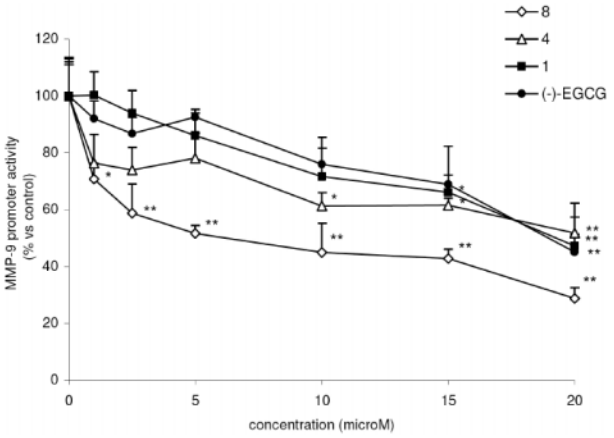


Figure 4. Concentration-inhibition curves for **8**, **4** and **1** on the MMP-9 promoter activity. Results were expressed as the inhibition of normalized luciferase activities versus control and represent the mean ± SD of three experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$ versus control.

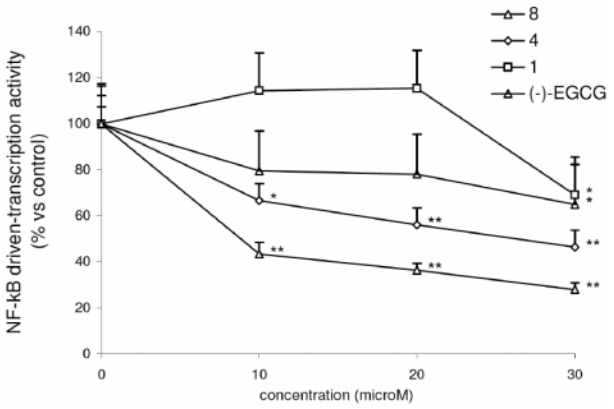


Figure 5. Effect of compounds **1**, **4** and **8** on NF-κB-driven transcription. Results were expressed as the inhibition of normalized luciferase activities versus control and represent the mean ± SD of two experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$ versus control.

transcription, indicating that the compounds may repress MMP-9 expression by interfering with this nuclear transcription factor pathway.

Discussion

Polyphenols are abundant components/micronutrients of the human diet that *in vitro* have been shown to profoundly affect ECM turnover by regulating expression and activity of MMPs, acting at pre- and post-transcriptional levels [7]. As shown in previous studies by ourselves and other authors, catechins modulate the function of MMP-9 at various steps including catalytic activation and transcription (via inhibition of promoter activity), either directly or by acting on regulators of transcription such as NF-κB [31], the MAPK family, and AP1 [10].

The study of the structural requirements necessary for a molecule to interact with a specific target may supply valuable information for the molecular modeling and design of novel inhibitors. SAR studies on catechins and related compounds have mainly focused on the structural requirements for radical scavenger and antioxidant activity [32–35]. However, polyphenols may affect many biological activities not only through their antioxidant effect, but also by interacting with specific molecular targets in the cell machinery. Elements that may be crucial for the interaction of polyphenols with molecular targets could be the presence of the galloyl group [11], the number and the position of hydroxyl groups on the flavan skeleton [17, 18] and the stereochemistry of the C ring [15]. Synthetic analogues of (–)-EGCG have been studied to understand the structure-function relationship of EGCG and to identify relevant mechanisms of the chemopreventive action of EGCG [36–37].

In this work we synthesized ten catechin derivatives characterized by a selective removal of phenolic hydroxyl groups. New flavan-3-ols characterized by an A ring deprived of phenolic hydroxyl groups, as well as new ester-type catechins, were obtained. Compounds **1,** **4** and **6** occur naturally in plants; in particular compound **4,** robidanol-3-gallate, has been identified in the aerial parts of *Euphorbia palustris* and *E. stepposa* [38–39]. Compounds **2**, **3**, **7**, **8**, **9** and **10** are structures never found in plants, although compound **2** derives from afzelechin, widely occurring in plants, and compound **9** derives from oxykoaburagenin, a catechin previously found in *Enkianthus nudipes* [40]. Compound **5** is a novel structure and the correspondent catechin has never been isolated.

The synthesized compounds were tested for assessing the SAR for MMP-9 regulation both at pre- and post-transcriptional levels.

Our results indicate that (±)-GCG **1** and (±)-catechin-3-gallate **6** are characterized by a substitution pattern compatible with direct inhibition of MMP-9 activity (fig. 3). All the other compounds prepared were substantially less active, thus indicating that the hydroxyl group R_3 of the B ring, which has been removed in compound **6**, might not be involved in specific interactions with the enzyme.

Some compounds reduced MMP-9 secretion and gene expression, although at different levels. When the target was MMP-9 gene transcription, very surprisingly, compound **8**, in which all the hydroxyl groups have been removed, was the most active with an IC_{50} of 4.2 μM. The effect was much less pronounced for the other catechins (five fold lower compared to **8**). For all the tested compounds, gene transcription down-regulation was mirrored by a decrease in the amount of protein secreted by the macrophages, although compounds **3** and **5** did not

follow this pattern. At this stage of the research we have no explanation for this observation, although we cannot exclude some post-transcriptional effects. Compound **8** was the most active also in modulating NF-κB driven transcription, indicating that a very lipophylic compound may have a preferential access at the nuclear level. Whether this is due to better permeation through cell membranes, or to a specific adaptability of this molecule to interact with the molecular target, still remains to be elucidated.

The results of the present study supply new insights into how catechin derivatives can act at the transcriptional or at the enzyme level, indicating that the structural features required for the enzymatic inhibition are different from those necessary for the down-regulation of gene expression. These data may provide new tools for designing potent and selective agents for the modulation of MMP-9 activity and gene expression.

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