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Research Article

Design, Synthesis and Biological Evaluation of Caffeic Acid Amides as Selective MMP-2 and MMP-9 Inhibitors

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT A series of caffeic acid amides with extended P1′ groups were synthesized and tested for their inhibitory activities on matrix metalloproteinase (MMP)-1, MMP-2, and MMP-9. Compound *3f* showed considerable inhibitory activities against MMP-2, MMP-9, and best selectivity over MMP-1. Preliminary structure–activity relationship analysis and docking studies indicated that caffeic acid amides with electron-donating groups at *p*-position of amino phenyl group showed better inhibitory activities and selectivity than those with electron-withdrawing groups. The findings of this study would provide information for the exploitation and utilization of caffeic acid as MMP inhibitor for metastatic tumor treatment. Drug Dev Res 73 : 343–351, 2012.

Key words: metrix metalloproteinase; caffeic acid; tumor; structure–activity relationship

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in the breakdown of components of the extracellular matrix that facilitates connective tissue remodeling [Nagase and Woessner, 1999]. The latter process is important in embryonic development, pregnancy, growth, and wound healing. Normally, MMP activity is tightly controlled by the balance between synthesis of active enzyme and the presence of endogenous inhibitors, e.g., the tissue inhibitor of metalloproteinases [Nagase and Woessner, 1999]. This balance is lost in tumor progression, where an increased expression of certain MMPs accompanies the passage from a benign to malignant phenotype that is believed to be involved in metastatic tumor dispersion and angiogenesis [MacDougall and Matrisian, 1995; Stetler-Stevenson, 1999].

MMPs are produced and secreted as inactive zymogens in the extracellular matrix from both tumor cells and surrounding stromal cells that are stimulated by the tumor. Two of these, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), appear to play a key role in these processes [Crabbe et al., 1994; Aimes and Quigley, 1995].

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In the past, some potent "broad-spectrum" MMP inhibitors (MMPIs) have been tested against tumors [Ray and Stetler-Stevenson, 1996] and some entered clinical trials, but none was approved for human use. One problem was the design of the clinical trials. MMPIs were efficacious in animal models of early stage disease but were tested in late-stage disease in humans [Overall and López-Otín, 2002]. Because they were developed as cytostatic agents, they may be used in a prolonged therapy, and therefore their bioavailability and slow long-term toxicity are key [Hodgson, 1995]. Another problem was a lack of selectivity with compounds such as CGS-27023A (**1**) (Ciba Geigy, Summit, NJ, USA) and BMS-275291 (**2**) (Bristol-Myers Squibb, Wallingford, CT, USA) (Fig. 1) showing a severe musculoskeletal syndrome, with fibroproliferative effects in the joint capsule of the knees [Hutchinson et al., 1998; Holmbeck et al., 1999; Steward, 1999]. These effects are thought to occur via impairment of normal tissue remodeling controlled by MMP-1 [Dahlberg et al., 2000]. For these reasons, a lack of activity with respect to MMP-1 is considered an important factor in reducing some of the side effects found for "nonselective" MMPIs [Scatena, 2000].

Selectivity could potentially be achieved by taking advantage of differences in size of the S1′ pocket among the various MMPs. From X-ray crystallographic, nuclear magnetic resonance (NMR) analysis, and homology modeling, MMPs may be classified into two broad structural classes: those with a relatively deep S1′ pocket (MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13) and those with a shallow S1′ pocket (MMP-1 and MMP-7) [Gooley et al., 1994; Lovejoy et al., 1994]. Consequently, incorporation of an extended P1′ group can lead to selective inhibition, whereas the presence of smaller P1′ groups generally leads to broad-spectrum inhibition.

Considering these data, many efforts have been directed to develop a more selective second generation of inhibitors against the specific MMPs believed to be involved in the different pathologies [Li et al., 2009]. MMPIs may also be derived from natural resources, e.g., herbs, plants, fruits, and other agriculture products have been highlighted in recent years.

Caffeic acid (**3**) (Fig. 2), which is found in fruits, vegetables, wine, olive oil, and coffee [Park et al., 2005], has been shown to inhibit the activity of MMP-9 with IC_{50} values of 10–20 nM. Furthermore, caffeic acid phenylester (**4**) (Fig. 2), extracted from honeybee propolis [Grunberger et al., 1988] and synthesized by esterification of CA [Nagaoka et al., 2002], selectively inhibited MMP-2, and MMP-9 but not MMP-1, MMP-3, and MMP-7 [Chung et al., 2004].

However, the ester groups in caffeic acid are metabolically labile and thus limited in use [Nakawaza and Ohsawa, 1998; Graefe and Veit, 1999; Celli et al., 2004]. Several modified caffeic acid amides have more stable characteristics [Rajan et al., 2001].

These findings prompted us to synthesize a series of corresponding caffeic acid amides with extended P1′ group for the purpose to investigate their selective inhibitory on MMP-2 and MMP-9, based on their inhibitory activities on MMP-1, MMP-2, and MMP-9, and structure–activity relationship (SAR) analysis was conducted. The results of this study may be useful to researchers attempting to find new potential caffeic acid derivatives as antitumor progression agents.

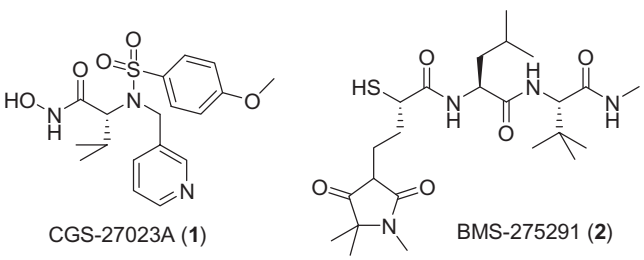

Fig. 1. Chemical structures of CGS-27023A (**1**) and BMS-275291 (**2**).

MATERIALS AND METHODS

Synthesis

Reagents and solvents were purchased from commercial sources and used without further purification unless otherwise specified. Air- and moisture-sensitive liquids and solutions were transferred via syringe or stainless steel cannula. Organic solutions were concentrated by rotary evaporation below 45°C at approximately 20 mm Hg. All nonaqueous reactions were carried out under anhydrous conditions using flame-dried glassware within an argon atmosphere in dry,

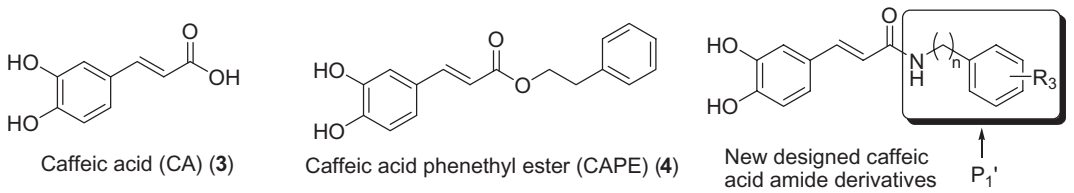

Fig. 2. Chemical structures of caffeic acid, caffeic acid phenethyl ester, and our further designed caffeic acid amide derivatives.

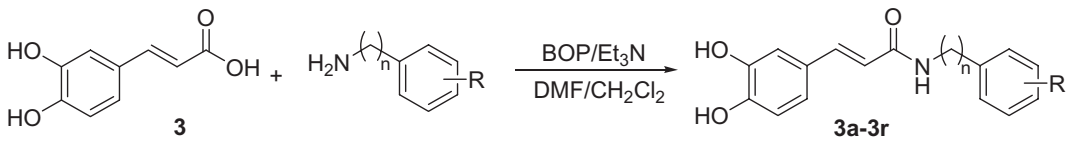

Fig. 3. Synthesis of compounds *3a–3r* using BOP as a condensing reagent.

TABLE 1. The Structures, Reaction Time, and Yields of Compounds *3a–3r*

Entry	R	*n*	Time	Prod. (yield)	Entry	R	*n*	Time	Prod. (yield)
1	H	0	8 h	*3a* (78)	10	2-CH_3	0	6 h	*3j* (84)
2	H	1	8 h	*3b* (83)	11	3-CH_3	0	6 h	*3k* (84)
3	H	2	8 h	*3c* (82)	12	4-CH_3	0	6 h	*3l* (86)
4	2-OH	0	6 h	*3d* (85)	13	2-F	0	7 h	*3m* (82)
5	3-OH	0	6 h	*3e* (84)	14	3-F	0	7 h	*3n* (75)
6	4-OH	0	6 h	*3f* (85)	15	4-F	0	7 h	*3o* (81)
7	2-OCH_3	0	6 h	*3g* (84)	16	2-Cl	0	7 h	*3p* (80)
8	3-OCH_3	0	6 h	*3h* (83)	17	3-Cl	0	7 h	*3q* (76)
9	4-OCH_3	0	6 h	*3i* (84)	18	4-Cl	0	7 h	*3r* (82)

freshly distilled solvents, unless otherwise noted. Yields referred to chromatographical seperation, unless otherwise stated. Reactions were monitored by thin-layer chromatography carried out on 0.15~0.20 mm Yantai silica gel plates (RSGF 254, Yantai Chemical Industry Research Institute, Yantai, Shandong Province, China) using UV light as the visualizing agent. Chromatography was performed on Qingdao silica gel (160~200 mesh, Qingdao Banke separation materials Company Limited, Qingdao, Shandong Province, China) using petroleum ether (60~90) and ethyl acetate as the eluanting solvent. 1H NMR spectra were obtained using a Bruker AV-300 (300 MHz) or Bruker AV-500 (500 MHz) (Bruker Corporation, Karlsruhe, Germany). Chemical shifts were recorded in parts per million downfield from tetramethylsilane. *J*-values were given in Hertz (Hz). Abbreviations used were singlet (s), doublet (d), triplet (t), quartet (q), broad (b), and multiplet (m). Electrospray ionization tandem mass spectrometry (ESI-MS) was recorded on a Waters Synapt high definition mass spectrometry (HDMS) spectrometer.

Using benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) as a condensing reagent, the free acid reacted with a variety of aniline in N,N-dimethylformamide (DMF) and CH_2Cl_2 (Fig. 3) to give the corresponding amide products in good yield (Table 1) [Fu et al., 2010]. 1H NMR and ESI-MS spectra were consistent with the assigned structures. Compounds *3a–3c* were acid anilides with the number of methylene were 0, 1, and 2, respectively. Compounds *3d–3r* were acid anilides with a single different substituent (OH, OCH_3, CH_3, F, or Cl) in *o*-, *m*-, or *p*-position of benzene ring (Table 1).

EXPERIMENTS

General Synthetic Method of Caffeic Acid Amides

To a stirring solution of substituted cinnamic acid (5 mmol) dissolved in DMF (20 ml) was added triethylamine (0.7 ml, 5 mmol) at 0°C. Then the amine (5 mmol) was added followed by a solution BOP (5 mmol) dissolved in CH_2Cl_2 (10 ml). The mixture was allowed to warm to room temperature and stirred for another 6–8 h, and then the reaction mixture was diluted with a large amount of ethyl acetate and washed with water and brine. The ethyl acetate layer was dried over $MgSO_4$, filtered and concentrated, and the crude material was purified by column chromatography (eluent: ethyl acetate–petroleum ether).

(E)-3-(3,4-dihydroxyphenyl)-N-phenylacrylamide (*3a*)

Yield 78% [Fu et al., 2010]. 1H NMR (dimethyl sulfoxide-d_6 (DMSO-d_6), 500 MHz) δ: 6.53 (d, J = 15.5 Hz, 1H), 6.77 (d, J = 7.5 Hz, 1H), 6.91 (dd, J_1 = 2 Hz, J_2 = 8.0 Hz, 1H), 7.00 (d, J = 2 Hz,1H), 7.04 (m, 1H), 7.31 (m, 2H), 7.40 (d, J = 15.5 Hz, 1H), 7.68 (d, J = 7.5 Hz, 1H), 7.71 (m, 1H), 9.13 (s, 1H), 9.40 (s, 1H), 10.02 (s, 1H). ESI-MS: m/z 256 $[M + H]^+$, 278 $[M + Na]^+$.

(E)-N-benzyl-3-(3,4-dihydroxyphenyl)acrylamide (*3b*)

Yield 83% [Rajan et al., 2001]. 1H NMR (DMSO-d_6, 500 MHz) δ: 4.38 (d, J = 6 Hz, 2H), 6.38 (d, J = 15.5 Hz, 1H), 6.74 (d, J = 8.0 Hz, 1H), 6.83 (dd, J_1 = 2 Hz, J_2 = 8.0 Hz, 1H), 6.94 (d, J = 2 Hz,1H), 7.27 (m, 6H), 8.44 (m, 1H), 9.07 (s, 1H), 9.30 (s, 1H). ESI-MS: m/z 270 $[M + H]^+$, 292 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-phenethylacrylamide (*3c*)

Yield 82% [Yang et al., 2010]. 1H NMR (DMSO-d_6, 500 MHz) δ: 2.76 (m, 2H), 3.39 (m, 2H), 6.31 (d, J = 15.5 Hz, 1H), 6.73 (d, J = 8.0 Hz, 1H), 6.82 (dd, J_1 = 2 Hz, J_2 = 8.0 Hz, 1H), 6.94 (d, J = 2 Hz,1H), 7.23 (m, 4H), 7.29 (m, 2H), 8.05 (m, 1H), 9.06 (s, 1H), 9.29 (s, 1H). ESI-MS: m/z 284 $[M + H]^+$, 306 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-(2-hydroxyphenyl) acrylamide (*3d*)

Yield 85% [Hung et al., 2005]. 1H NMR (DMSO-d_6, 500 MHz) δ: 6.79 (m, 2H), 6.87 (m, 1H), 6.94 (m, 2H), 7.02 (d, J = 2 Hz,1H), 7.39 (d, J = 16.0 Hz, 1H), 7.81 (d, J = 7.5 Hz, 2H), 7.97 (m, 1H), 9.09 (s, 1H), 9.41 (s, 1H), 9.46 (s, 1H), 9.93 (s, 1H). ESI-MS: m/z 272 $[M + H]^+$, 294 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-(3-hydroxyphenyl) acrylamide (*3e*)

Yield 84% [Hung et al., 2005]. 1H NMR (DMSO-d_6, 500 MHz) δ: 6.44 (d, J = 7.0 Hz, 1H), 6.53 (d, J = 15.5 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.90 (dd, J_1 = 2.0 Hz, J_2 = 8.0 Hz, 1H), 7.00 (d, J = 2.0 Hz, 1H), 7.05 (m, 2H), 7.27 (s, 1H), 7.37 (d, J = 15.5 Hz, 1H), 9.13 (s, 1H), 9.33 (s, 1H), 9.39 (s, 1H), 9.88 (s, 1H). ESI-MS: m/z 272 $[M + H]^+$, 294 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-(4-hydroxyphenyl) acrylamide (*3f*)

Yield 85% [Seo et al., 2005]. 1H NMR (DMSO-d_6, 500 MHz) δ: 6.48 (d, J = 16.0 Hz, 1H), 6.71 (d, J = 8.0 Hz, 2H), 6.76 (d, J = 8.0 Hz, 1H), 6.88 (dd, J_1 = 2.0 Hz, J_2 = 8.0 Hz, 1H), 6.98 (d, J = 2 Hz, 1H), 7.34 (d, J = 16.0 Hz, 1H), 7.46 (d, J = 9.0 Hz, 2H), 9.11 (s, 1H), 9.14 (s, 1H), 9.36 (s, 1H), 9.77 (s, 1H). ESI-MS: m/z 272 $[M + H]^+$, 294 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-(2-methoxyphenyl) acrylamide (*3g*)

Yield 84% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 500 MHz) δ: 3.86 (s, 3H), 6.76 (d, J = 8.0 Hz, 1H), 6.86–6.93 (m, 3H), 7.02 (d, J = 2.0 Hz, 1H), 7.03–7.08 (m, 2H), 7.36 (d, J = 15.5 Hz, 1H), 8.13 (d, J = 8.0 Hz, 1H), 9.06 (s, 1H), 9.17 (s, 1H), 9.39 (s, 1H). ESI-MS: m/z 286 $[M + H]^+$, 308 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-(3-methoxyphenyl) acrylamide (*3h*)

Yield 83% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 500 MHz) δ: 3.74 (s, 3H), 6.52 (d, J = 15.5 Hz, 1H), 6.62–6.64 (m, 1H), 6.78 (d, J = 8.0 Hz, 1H), 6.90 (dd, J_1 = 2.0 Hz, J_2 = 8.0 Hz, 1H), 7.00 (d, J = 2.0 Hz, 1H), 7.18–7.23 (m, 2H), 7.38–7.41 (m, 2H), 9.14 (s, 1H), 9.40 (s, 1H), 10.01 (s, 1H). ESI-MS: m/z 286 $[M + H]^+$, 308 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-(4-methoxyphenyl) acrylamide (*3i*)

Yield 84% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 500 MHz) δ: 3.73 (s, 3H), 6.16 (d, J = 15.5 Hz, 1H), 6.75 (d, J = 8.0 Hz, 1H), 6.95 (dd, J_1 = 2 Hz, J_2 = 8.0 Hz, 1H), 7.01 (d, J = 2.0 Hz, 1H), 7.53–7.56 (m, 3H), 7.72 (d, J = 8.0 Hz, 2H), 9.07 (s, 1H), 9.47 (s, 1H). ESI-MS: m/z 286 $[M + H]^+$, 308 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-o-tolylacrylamide (*3j*)

Yield 84% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 500 MHz) δ: 2.24 (s, 3H), 6.67 (d, J = 15.5 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.90 (dd, J_1 = 2 Hz, J_2 = 8.0 Hz, 1H), 7.01 (d, J = 2 Hz, 1H), 7.07 (m, 1H), 7.17 (m, 1H), 7.22 (d, J = 8.0 Hz, 1H), 7.38 (d, J = 15.5 Hz, 1H), 7.57 (d, J = 8.0 Hz, 1H), 9.09 (s, 1H), 9.28 (s, 1H), 9.38 (s, 1H). ESI-MS: m/z 270 $[M + H]^+$, 292 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-m-tolylacrylamide (*3k*)

Yield 84% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 500 MHz) δ: 2.29 (s, 3H), 6.53 (d, J = 15.5 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.90 (dd, J1 = 2 Hz, J2 = 8.0 Hz, 1H), 7.01 (d, J = 2 Hz, 1H), 7.19 (m, 1H), 7.38 (d, J = 15.5 Hz, 1H), 7.51 (m, 3H), 9.13 (s, 1H), 9.39 (s, 1H), 9.94 (s, 1H). ESI-MS: m/z 270 $[M + H]^+$, 292 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-p-tolylacrylamide (*3l*)

Yield 86% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 500 MHz) δ: 2.26 (s, 3H), 6.52 (d, J = 15.5 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.89 (dd, J_1 = 2 Hz, J_2 = 8.0 Hz,

1H), 6.99 (d, J = 2 Hz,1H), 7.11 (d, J = 8.0 Hz, 2H), 7.36 (d, J = 15.5 Hz, 1H), 7.56 (d, J = 8.0 Hz, 2H), 9.12 (s, 1H), 9.38 (s, 1H), 9.93 (s, 1H). ESI-MS: m/z 270 $[M + H]^+$, 292 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-(2-fluorophenyl) acrylamide (*3m*)

Yield 82% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 500 MHz) δ: 6.76–6.79 (m, 2H), 6.90–6.92 (dd, $J_1 = 2$ Hz, $J_2 = 8.0$ Hz, 1H), 7.01 (d, J = 2 Hz, 1H), 7.11–7.19 (m, 2H), 7.24–7.28 (m, 1H), 7.41 (d, J = 15.5 Hz, 1H), 8.07–8.10 (m, 1H), 9.13 (s, 1H), 9.42 (s, 1H), 9.76 (s, 1H). ESI-MS: m/z 274 $[M + H]^+$, 296 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-(3-fluorophenyl) acrylamide (*3n*)

Yield 75% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 300 MHz) δ: 6.50 (d, J = 15.6 Hz, 1H), 6.76 (d, J = 8.1 Hz, 1H), 6.90 (dd, $J_1 = 2.1$ Hz, $J_2 = 8.1$ Hz, 1H), 6.99 (d, J = 2.1 Hz, 1H), 7.37–7.39 (m, 2H), 7.65–7.72 (m, 3H), 9.15 (s, 1H), 9.42 (s, 1H), 10.23 (s, 1H). ESI-MS: m/z 274 $[M + H]^+$, 296 $[M + Na]^+$.

(E)-3-(3,4-dihydroxyphenyl)-N-(4-fluorophenyl) acrylamide (*3o*)

Yield 81% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 300 MHz) δ: 6.48 (d, J = 15.6 Hz, 1H), 6.75 (d, J = 8.1 Hz, 1H), 6.88 (dd, $J_1 = 2.1$ Hz, $J_2 = 8.1$ Hz, 1H), 6.98 (d, J = 2.1 Hz, 1H), 7.11–7.16 (m, 2H), 7.39 (d, J = 15.6 Hz, 1H), 7.67–7.72 (m, 2H), 9.13 (s, 1H), 9.40 (s, 1H), 10.08 (s, 1H). ESI-MS: m/z 274 $[M + H]^+$, 296 $[M + Na]^+$.

(E)-N-(2-chlorophenyl)-3-(3,4-dihydroxyphenyl) acrylamide (*3p*)

Yield 80% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 300 MHz) δ: 6.75–6.80 (m, 2H), 6.90 (dd, $J_1 = 1.8$ Hz, $J_2 = 8.1$ Hz, 1H), 7.01 (d, J = 1.8 Hz, 1H), 7.13–7.18 (m, 1H), 7.51 (d, J = 15.5 Hz, 1H), 7.63–7.66 (m, 1H), 7.68–7.72 (m, 1H), 7.88–7.91 (m, 1H), 9.11 (s, 1H), 9.43 (s, 1H), 9.48 (s, 1H). ESI-MS: m/z 290 $[M + H]^+$, 312 $[M + Na]^+$.

(E)-N-(3-chlorophenyl)-3-(3,4-dihydroxyphenyl) acrylamide (*3q*)

Yield 76% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 300 MHz) δ: 6.48 (d, J = 15.6 Hz, 1H), 6.76 (d, J = 8.1 Hz, 1H), 6.90 (dd, $J_1 = 2.1$ Hz, $J_2 = 8.1$ Hz, 1H), 6.99 (d, J = 2.1 Hz, 1H), 7.06–7.10 (m, 2H), 7.30–7.35 (m, 1H), 7.37–7.43 (m, 1H), 7.47–7.55 (m, 1H), 7.90 (m, 1H), 9.15 (s, 1H), 9.44 (s, 1H), 10.21 (s, 1H). ESI-MS: m/z 290 $[M + H]^+$, 312 $[M + Na]^+$.

(E)-N-(4-chlorophenyl)-3-(3,4-dihydroxyphenyl) acrylamide (*3r*)

Yield 82% [Fu et al., 2010]. 1H NMR (DMSO-d_6, 500 MHz) δ: 6.51 (d, J = 15.5 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.91 (dd, $J_1 = 1.5$ Hz, $J_2 = 8.0$ Hz, 1H), 7.01 (d, J = 1.5 Hz, 1H), 7.36–7.43 (m, 3H), 7.70–7.73 (m, 2H), 9.14 (s, 1H), 9.42 (s, 1H), 10.16 (s, 1H). ESI-MS: m/z 290 $[M + H]^+$, 312 $[M + Na]^+$.

BIOLOGICAL SCREENING

MMP-1, MMP-2, and MMP-9 Inhibition Assay

The acid amides were assayed for the inhibition against MMP-1, MMP-2, and MMP-9 in 96-well microtiter plates using succinylated gelatin as the substrate [Baragi et al., 2000]. The compound and enzyme were dissolved in sodium borate buffer (pH 8.5, 50 mM) and incubated at 37°C for 30 min. The substrate was added and incubated at 37°C for another 60 min. The 100% and blank groups were also used, in which the 100% group contained no compound and the blank group contained only the enzyme. Then 0.03% picrylsulfonic acid solution was added and incubated at room temperature for additional 20 min. The resulting solutions were measured under 450 nm to gain optical density 450 (OD_{450}) values, which were then used to calculate the inhibitory rates by $[OD_{450}(100\%) - OD_{450}(\text{compound})]/[OD_{450}(100\%) - OD_{450}(\text{blank})] \times 100\%$. The IC_{50} values were obtained from the previous inhibitory rates using OriginPro 7.5 software (OriginLab Corporation, Hampton, MA, USA).

RESULTS AND DISCUSSION

MMP-1, MMP-2, and MMP-9 inhibitory activity

Table 2 summarizes the results obtained in the assay for the inhibitory activities on MMP-1, MMP-2, and MMP-9; caffeic acid was used as the reference control, selectivity indices for MMP-2 and MMP-9 over MMP-1 are also reported, expressed as ratios of their inhibitory indices (Table 2). The results showed that these caffeic acid amides had no significant inhibition activities against MMP-1 supported by the fact that their IC_{50}s were greater than 1000 nM, thus confirming our strategy for designing MMP-2 and MMP-9 inhibitors (Table 2).

Among the synthesized amides, the most active compound was *3f*, its IC_{50} values on MMP-2 and

TABLE 2. The Inhibitory Activity of Compounds *3a–3r* Toward Some of the Principal MMPs, the MMP-2 Selectivity[a] and the MMP-9 Selectivity[a] (in Parentheses)

	IC_{50}[b] (nM)		
Product	MMP-1	MMP-2	MMP-9
3a	3,235.42 ± 28.19	8.05 ± 0.53 (402)	7.14 ± 0.58 (453)
3b	3,246.57 ± 27.32	8.22 ± 0.49 (395)	7.25 ± 0.51 (448)
3c	3,301.33 ± 30.14	8.20 ± 0.51 (403)	7.28 ± 0.60 (453)
3d	5,616.56 ± 42.89	5.09 ± 0.34 (1,103)	5.28 ± 0.49 (1,064)
3e	5,769.78 ± 40.47	1,587.17 ± 21.31 (4)	1,642.27 ± 22.43 (4)
3f	5,833.61 ± 42.16	3.28 ± 0.29 (1,779)	2.35 ± 0.12 (2,482)
3g	5,247.92 ± 39.28	5.27 ± 0.58 (996)	5.67 ± 0.43 (926)
3h	5,285.42 ± 41.85	1,679.56 ± 22.53 (3)	1,632.42 ± 21.38 (3)
3i	5,319.54 ± 43.79	3.42 ± 0.49 (1,555)	3.33 ± 0.42 (1,597)
3j	4,712.73 ± 35.84	5.97 ± 0.58 (789)	5.91 ± 0.53 (797)
3k	4,799.42 ± 37.92	1,721.82 ± 23.57 (3)	1,753.85 ± 22.61 (3)
3l	4,829.57 ± 41.47	3.55 ± 0.51 (1,360)	3.64 ± 0.49 (1,327)
3m	4,246.83 ± 40.91	8.92 ± 0.62 (476)	7.81 ± 0.66 (544)
3n	4,302.21 ± 38.67	1,820.53 ± 24.60 (2)	1,869.59 ± 22.70 (2)
3o	4,366.42 ± 39.52	8.31 ± 0.59 (525)	7.29 ± 0.68 (599)
3p	3,706.63 ± 30.19	9.11 ± 0.72 (407)	8.19 ± 0.77 (453)
3q	3,808.43 ± 31.24	1,873.02 ± 23.71 (2)	1,921.13 ± 25.71 (2)
3r	3,963.66 ± 31.97	8.95 ± 0.73 (443)	7.89 ± 0.69 (502)
CA	238.91 ± 2.22	24.26 ± 0.42 (10)	21.22 ± 1.31 (11)

[a]Selectivity for MMP-1 over each of the other MMPs, is expressed as the ratio of the IC_{50} value for MMPs over the value for MMP-1.
[b]IC_{50} values are mean of four experiments, standard deviation is given.

MMP-9 were 3.28 and 2.35 nM, respectively. Furthermore, compound *3f* also showed the highest selectivity profile (MMP-1/MMP-2 ratio was 1,779, MMP-1/MMP-9 ratio was 2,482). The *ortho* isomer of *3f*, compound *3d*, had a good inhibitory activity on MMP-2 (IC_{50} = 5.09 nM) and MMP-9 (IC_{50} = 5.28 nM). While its *meta* isomer, compound *3e*, had weak activity, (IC_{50} values for MMP-2 and MMP-9 were 1587.17 and 1642.27 nM, respectively). Replacing the hydroxyl group with electron-donating groups such as methoxy group or methyl group resulted in little effect on the inhibitory activity against MMP-2, MMP-9, and selectivity over MMP-1. For example, the *p*-MeO substituted compound *3i* (MMP-2 IC_{50} = 3.42 nM, MMP-9 IC_{50} = 3.33 nM) and the *p*-Me substituted compound *3l* (MMP-2 IC_{50} = 3.55 nM, MMP-9 IC_{50} = 3.64 nM) proved to be slightly less active than *3f*. However, replacing the hydroxyl group with electron-withdrawing groups such as fluorine or chlorine resulted in decreased inhibitory activity against MMP-2, MMP-9, and selectivity over MMP-1. For example, the *p*-F substituted compound *3o* (MMP-2 IC_{50} = 8.31 nM, MMP-9 IC_{50} = 7.29 nM) and the *p*-Cl substituted compound *3r* (MMP-2 IC_{50} = 8.95 nM, MMP-9 IC_{50} = 7.89 nM) exhibited an inhibitory activity about three times lower than *3f* (MMP-2 IC_{50} = 3.28 nM, MMP-9 IC_{50} = 2.35 nM). In addition, replacement of the hydroxyanil (*3f*) with aniline (*3a*, MMP-2 IC_{50} = 8.05 nM, MMP-9 IC_{50} = 7.14 nM), benzylamine (*3b*, MMP-2 IC_{50} = 8.22 nM, MMP-9 IC_{50} = 7.25 nM), and phenylethylamine (*3c*, MMP-2 IC_{50} = 8.20 nM, MMP-9 IC_{50} = 7.28 nM) led to slightly less inhibitory activity on MMP-2 and MMP-9 than *3f*.

Docking Studies

In the MMP inhibition tests, *3f* showed the strongest inhibitory activity on MMP-2 and MMP-9, and the highest selectivity against MMP-1, so the caffeic acid and *3f* were selected for the subsequent molecular docking experiment with MMP-2 and MMP-9. The docking studies of caffeic acid (Fig. 4) showed that the carboxylic acid group in caffeic acid could occupy the deep S1′ pocket in MMP-2 and MMP-9, and the carbonyl group chelated the active site zinc ion with a distance of 2.347 Å in MMP-2 and 2.210 Å in MMP-9, respectively. The docking studies of *3f* with MMP-2 and MMP-9 (Fig. 5) indicated that *3f* interacted well with these two MMPs. The MMP-2 docking of *3f* showed that the *para*-hydroxy aniline group in *3f* occupied well the deep S1′ pocket of MMP-2, and the carbonyl group chelated the active site zinc ion with a distance of 2.296 Å. The docking studies of *3f* with MMP-9 showed that the *para*-hydroxy aniline group in *3f* occupied well the deep S1′ pocket of MMP-9, and the carbonyl group chelated the active site zinc ion with a distance of 3.774 Å. These results indicated that compound *3f* interacted well with MMP-2 and MMP-9 active site,

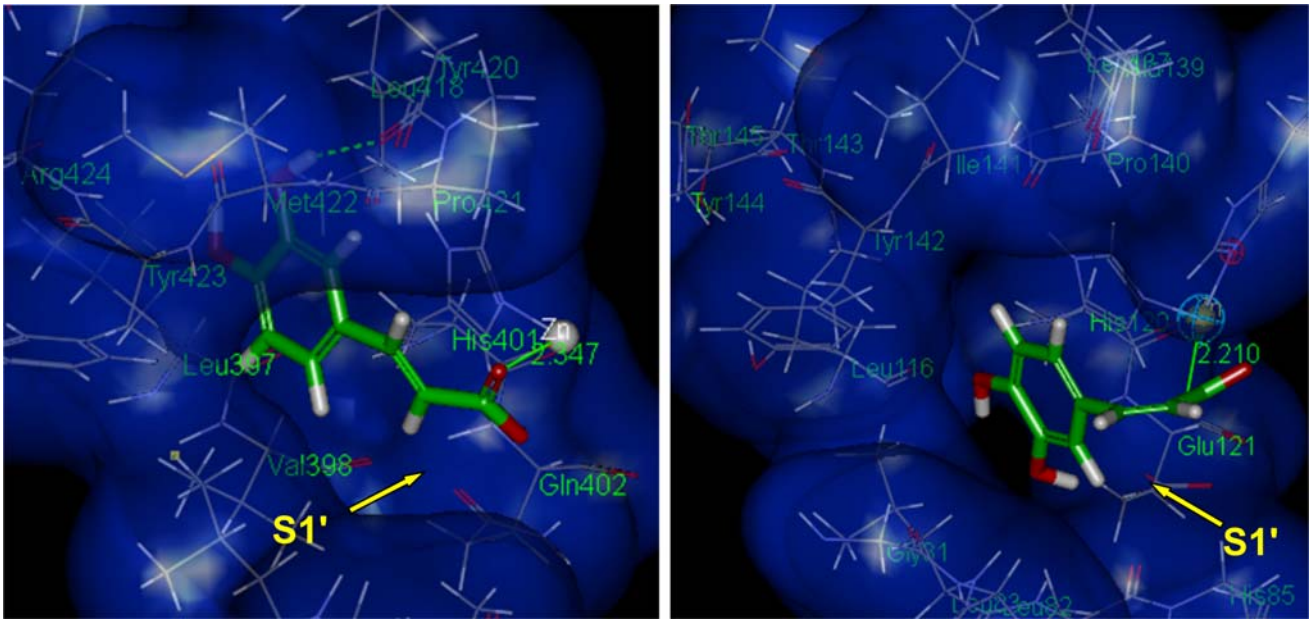

Fig. 4. Docking result of caffeic acid with MMP-2 (left) and MMP-9 (right). [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

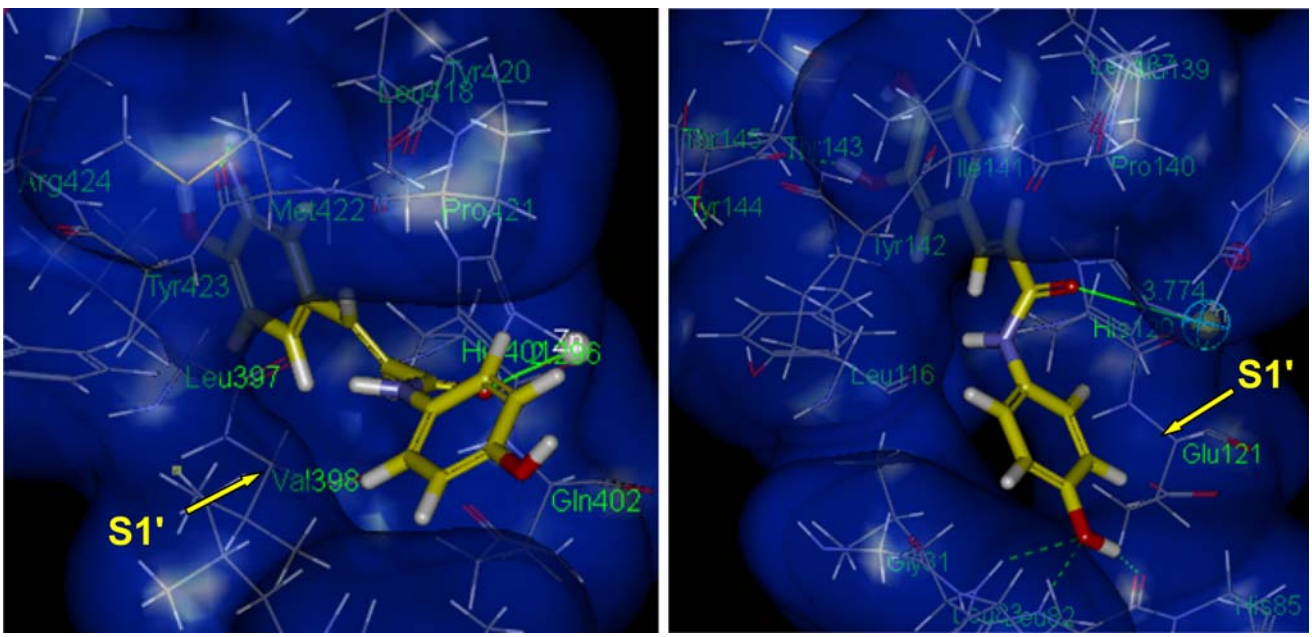

Fig. 5. Docking result of compound *3f* with MMP-2 (left) and MMP-9 (right). [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

especially the deep S1′ pocket and zinc ion, consistent with MMP-2 and MMP-9 assay results.

Discussion and SAR

Although the synthesized acid anilides exhibited better inhibitory activity against MMP-2 and MMP-9, their IC_{50}s differ significantly. For example, among the acid anilides *3a–3c* with no substitutions on the amino phenyl group, anilides with no carbon atom between the amide and the phenyl group showed more inhibitory activities on MMP-2 and MMP-9 than those with one carbon atom or two carbon atoms between the amide and the phenyl group. Such as in the inhibitory

activity on MMP-2, compound *3a* with phenyl group showed a good potency with an IC_{50} was 8.05 nM, and replacing the phenyl group with the benzyl group and the phenylethyl group, as shown in compounds *3b* and *3c*, resulted in slightly lower inhibitory activity with their IC_{50}s were 8.22 and 8.20 nM, respectively.

Furthermore, among all the synthesized anilides, the most active compound was *3f* with one hydroxyl group at *p*-position, it showed the best inhibitory potency on MMP-2 and MMP-9 with its IC_{50}s were 3.28 and 2.35 nM, respectively. In addition, *3f* was the compound with the highest selectivity profile (MMP-1/MMP-2 ratio was 1,779, MMP-1/MMP-9 ratio was 2,482). In the series of caffeic acid anilides, replacement of the hydroxyl group of (*3f*) at *p*-position by electron-donating groups such as methoxy group (*3i*) or methyl group (*3l*) resulted in little effect on the inhibitory activity against MMP-2, MMP-9, and selectivity over MMP-1. However, replacement of the hydroxyl group of (*3f*) at *p*-position by electron-withdrawing groups such as fluorine (*3o*) or chlorine (*3r*) resulted in the decrease of inhibitory activity against MMP-2, MMP-9, and selectivity over MMP-1. These results suggested that compounds with electron-donating groups at *p*-position showed better inhibitory activities and selectivity than those with electron-withdrawing groups.

It should be mentioned that anilides with a single substitute at the *para*-positions of the benzene ring exhibited the most powerful inhibitory activities against MMP-2 and MMP-9, anilides with a substituent at *ortho*-positions showed less potent activites, whereas anilides with a substituent at *mata*-positions showed diminished activity. From this fact, it may be reasoned that the *para*-position was an essential point for the inhibition activities against MMP-2 and MMP-9, and substituents at the *ortho*-position decreased their inhibition, substituents at the *mata*-position showed no inhibitory activities. For example, when a hydroxyl group was introduced at *para*-position to form *3f*, the inhibitory activities against MMP-2 and MMP-9 was the most significant, and when a hydroxyl group was introduced at *ortho*-position to form *3d*, the activity decreased slightly. However, when a hydroxyl group was introduced at *mata*-position to form *3e*, the activity decreased significantly.

CONCLUSIONS

In this study, a series of acid amides *3a–3r* were synthesized, and through measurement using a fluorogenic substrate assay with human recombinant MMP-1, MMP-2, and MMP-9, compound *3f* showed considerable inhibitory activities against MMP-2, MMP-9, and best selectivity over MMP-1. Preliminary SAR analysis indicated that caffeic acid anilides with electron-donating groups at *para*-position of amino phenyl group showed better inhibitory activities and selectivity than those with electron-withdrawing groups. Therefore, the findings of this study would facilitate the design of chemical compounds with higher potency to serve as selective MMP-2 and MMP-9 inhibitors, and provide information for the exploitation and utilization of caffeic acid as MMPI for metastatic tumor treatment.

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