

Natural Bio-Drugs as Matrix Metalloproteinase Inhibitors: New Perspectives on the Horizon?[§]

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Abstract: The matrix metalloproteinases (MMPs), belonging to the family of proteolytic enzymes, are well-known for their ability to degrade the extracellular matrix, and are involved in many aspects of both physiological cellular processes and pathological situations, such as tumor growth, invasion and metastasis. MMPs have been considered prognostic factors in various types of cancer as well as promising targets for cancer therapy. Although preclinical studies of a number of different synthetic MMP inhibitors have been identified as cytostatic and anti-angiogenic agents and have begun clinical testing, the past years have produced a consistent number of disappointments and limited successes. In view of their specific implication in malignant tissues, several natural compounds were utilized, and the results were so satisfactory as to encourage several clinical trials in order to improve efficacy and to reduce the side effect profile. The natural protection against cancer has been receiving a great deal of attention, and the critical examination of previous studies shed light on new information about the source and function of MMPs, focusing the attention on the identification of MMP targets in tumors. This review discusses the current knowledge and research in the field of natural MMP inhibitor as innovative therapeutic intervention in cancer.

Keywords: Biodrugs, Cancer, Clinical Trials, Gelatinases, Matrix Metalloproteinases, Natural Compounds, Nutraceuticals, Protease Inhibitors, Therapeutic strategies.

[§]*Dedicated to the memory of my father*

("Bene qui latuit bene vixit", One who lived well, lived unnoticed; Ovid).

Mater artium necessitas. "Necessity is the mother of invention" (Apuleius)

INTRODUCTION

Cancer is one of the leading causes of death and disease world-wide [1]. At the same time, clinicians and researchers are focusing more on the concept of targeted therapies and the finding of new and relevant prognostic factors to better delineate treatment options in cancer patients. Clinicians use a number of factors to stratify cancer patients at diagnosis, in order to accurately define risk profiles and plan the most appropriate treatment. Along with patient and tumor characteristics (such as age at diagnosis, tumor size, and lymph node status), an increasing number of molecular prognostic factors, (named tumor "biomarkers"), are being developed for use in patient outcome and treatment determinations [2]. These markers must have some defining characteristics, such as ease of specimen collection and a reproducible assay that is rapid and inexpensive [3]. Although hundreds of these biomarkers have been reported, few are proving to be useful in the clinic. Large amounts of data have been obtained relative to matrix metalloproteinase (MMP) overexpression in various tumor types when compared to normal tissues [4], and several studies have provided evidence that certain MMPs in specific cancers can be useful as indicators of disease progression, thereby

improving treatment strategies and management of specific cancers [5].

THE MATRIX METALLOPROTEINASE FAMILY

The MMPs are a family of structurally and functionally related endoproteases that are collectively capable of degrading most of the components of the extracellular matrix (ECM) [3, 6]. These enzymes share common functional domains and activation mechanisms as they depend on Ca and Zn ions and are active at neutral pH. As shown in Fig. (1), the structure organization of all MMPs presents a pre-peptide sequence that directs their secretion in the extracellular environment and a pro-peptide domain that maintains them in their zymogenic form. The pro-peptide keeps the enzyme in an inactive/latent state through the interaction of a cysteine residue with the catalytic zinc. Complete activation is obtained when this interaction is disrupted by chaotropic agents or by cleavage of the propeptide by other MMPs or proteinases [7]. MMPs possess a variable hinge region that links the catalytic domain, to a hemopexin-like domain which may be involved in substrate specificity [3]. Moreover, the family of MMPs has evolved by incorporating and/or deleting structural and functional domains. For instance, two MMPs (known as gelatinases), present the insertion of three fibronectin type II repeats in the catalytic domain which are involved in substrate binding. Other MMPs, known as membrane type MMPs (MT-MMP), have a hydrophobic amino acid stretch that crosses the cell membrane docking them to the cell surface. Finally, some members of this ever-growing family contain in their

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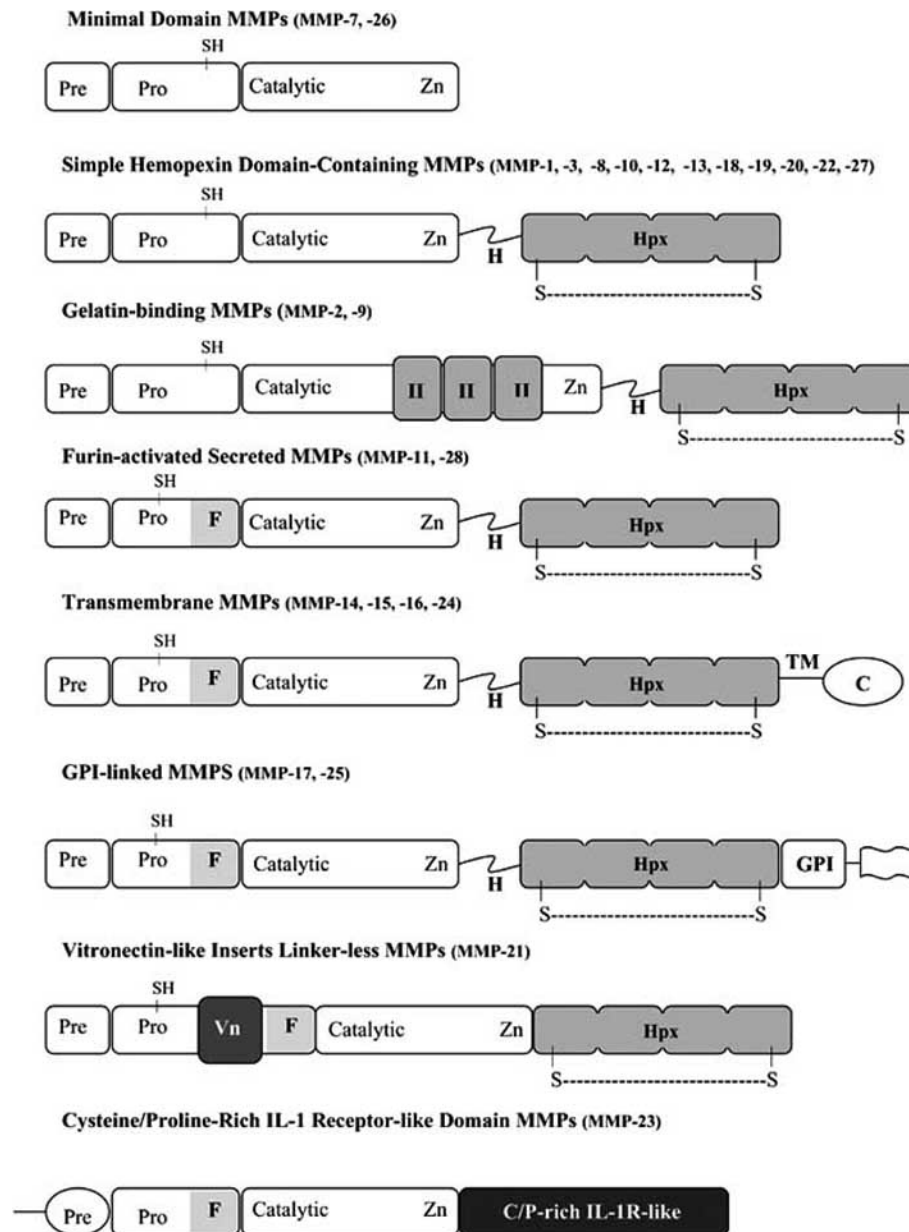


Fig. (1). Structure of human matrix metalloproteinases.

The signal pre-peptide directs the proenzyme for secretion. The propeptide contains a conserved sequence, in which the cysteine forms a covalent bond with the catalytic zinc to maintain the latency of pro-MMPs. Catalytic domain contains the highly conserved zinc binding site in which zinc is coordinated by histidines. The proline-rich hinge region links the catalytic domain to the hemopexin domain, which determines the substrate specificity of specific MMPs. The hemopexin domain is absent in matrilysins. Gelatinases contain repeats of fibronectin-type II domain inserted in the catalytic domain. MT-MMPs are characterized by transmembrane domains, containing a glycosylphosphatidyl-inositol anchor in the C-terminus of the molecule, which attach these MMPs to the cell surface. The presence of a furin cleavage site between the propeptide and catalytic domain makes these proenzymes susceptible to activation by intracellular furin convertases. The N-terminal signal anchor allows the MMP-23 (with different C-terminal domain) to anchor the Golgi complex.

Pre, signal sequence. **Pro**, propeptide with a free zinc-ligating thiol (SH) group. **Hpx**, hemopexin-like domain. **F**, furin-susceptible site. **Zn**, zinc-binding site. **II**, collagen-binding fibronectin type II inserts. **H**, hinge region. **TM**, trans-membrane domain. **C**, cytoplasmic tail. **GPI**, glycosylphosphatidylinositol-anchoring domain. **C/P**, cysteine/proline. **IL-1R**, interleukin-1 receptor.

prodomain a recognition motif for furin-like enzymes, whose cleavage activates MMPs [7].

MMP activity can be controlled at various levels: transcription, proteolytic activation of the zymogen form,

and inhibition of the active enzyme [8]. Most MMPs are expressed at low levels or not at all in resting-state adult tissues, but their expression can be rapidly stimulated by numerous cytokines and growth factors as well as by

physical cellular interaction [3, 6]. Activation of most MMPs via the "cysteine switch" mechanisms occurs outside the cell through the disruption of the interaction between the cysteine-sulphydryl group and the zinc ion, but several MMPs can be activated by intracellular furin-like serine proteases before reaching the cell surface. Most MMPs can also be activated by other MMPs or by serine proteases [9]. Due to the important regulative roles MMPs play, their activity must be tightly regulated and several endogenous inhibitors control their activity. The main endogenous inhibitor in tissue fluids is α_2 -macroglobulin, which forms a complex with MMP that gets successively removed by scavenger receptors [8]. Other molecules that can regulate MMP activity are thrombospondin-1 and -2 and the cell surface receptor known as reversion-inducing cysteine-rich protein with kazal motifs RECK [5]. However, the most important inhibitors are the tissue inhibitors of metalloproteinases (TIMPs). Up to now, five TIMPs have been identified as small molecules of 20-30 kDa that reversibly bind and block MMPs in a stoichiometric ratio. TIMPs differ in their specificity towards MMPs and in their pattern expression [10, 11].

MMPs are able to degrade practically all ECM components and can be classified according to their substrate specificity in collagenases, gelatinases, stromelysins and matrilysins Fig. (1)[8]. Owing to their wide proteolytic activity, these enzymes were initially considered only as "bulldozers" destroying ECM scaffold and chewing holes in the extracellular environment, but it soon became evident that these proteolytic enzymes had much more under their skin. In fact, it is now clear that MMPs not only control the homeostasis and remodeling of the ECM, but also influence many biological pathways during developmental and physiological processes [6, 12]. MMPs regulate cell migration due to their proteolytic activity and also because their cleavage can generate fragments with new functions. For example, cleavage of laminin-5 and collagen type IV makes accessible cryptic sites that promote migration, thus favoring cancer cell motility [13]. Evidence suggests that MMPs also have "shedase" activity, giving them the ability to release bioactive molecules from the cell surface, and these non-ECM substrates range from growth factor precursors and binding proteins to cell surface adhesion receptors [5]. Degradation of insulin-like growth factor binding protein (IGF-BP) releases IGF; cleavage of perlecan releases fibroblast growth factor [13, 14]; proteolysis of decorin upregulates the bioavailability of TGF- β ; cleavage of E-cadherin and the extracellular domain of the hyaluronan receptor CD44 leads to increased invasive behavior, cleavage of the α_5 and the β_1 -integrin chain, cleavage of ICAM-1, release of tumor necrosis factor (TNF) from the cell surface and release of Fas ligand (FasL) induces apoptosis and cleavage of the neutrophil chemoattractant interleukin-8 and monocyte chemoattractant protein-3 (MCP-3) [reviewed in 5, 9, 12, 13].

The wide number of MMP substrates increases the biological processes in which these proteolytic enzymes can have important regulative roles [14], making MMPs key effectors in many physiological events, but also evidencing that a dysregulated/uncontrolled MMP expression may lead to pathological states such as tumor development and

metastasis, thus being ideal candidates for molecules that mediate host/tumor interactions [5].

MATRIX METALLOPROTEINASES IN CANCER PROGRESSION

MMPs are overexpressed in multiple tumor types when compared to normal tissues [5, 13]. The upregulation of the MMPs may be a secondary effect of the remodeling of the matrix and/or growth characteristics of the tumor [9]. However, in cases in which increased MMP levels have been shown to be strong indicators of a negative prognosis, it is more likely that targeting those enzymes will impact tumor progression; several studies pointed to the possible use of MMPs in the future to augment treatment strategies in specific cancers [4, 5].

MMPs were classically thought to contribute to tumor metastasis via their matrix degrading activity, but in recent years, studies have implicated MMPs at virtually all stages of tumor progression from initial development of the tumor, growth, angiogenesis, invasion, and metastasis and growth at the secondary site [13]. There are several ways in which MMPs can increase tumor cell proliferation: first of all, MMPs can release the cell-membrane-bound precursors of some growth factors; they can render bioavailable peptide growth factors that are sequestered by ECM proteins and moreover they can control proliferation signals through integrins [14]. MMPs also confer anti-apoptotic characteristics to cancer cells as can be seen by the release of FasL [12]. For tumor cells to continue growing and to migrate to distant sites, the formation of new blood vessels is a fundamental step and many studies with endogenous and synthetic inhibitors indicate the central role MMPs occupy in this process [15]. MMPs can favor new blood vessel sprouting by simply eliminating physical barriers through the degradation of ECM structural components, or by the generation of pro-angiogenic factors. In fact, it has been demonstrated that the cleavage of collagen type IV exposes a cryptic binding site essential for endothelial cell migration increasing the bioavailability of the pro-angiogenic vascular endothelial growth factor [16]. Strangely enough, MMPs can also downregulate new vessel formation through the generation of angiogenesis inhibitors. In fact, the cleavage of plasminogen and the degradation of the basement membrane collagen type XVIII generates endostatin and angiostatin, both molecules possessing inhibitory capacity towards angiogenesis [17, 18]. For tumor-cells to migrate and metastasize, it is necessary to break down several ECM barriers, and cell movement is strongly associated with proteolysis and requires bi-directional interactions between cell and ECM [9]. MMPs take part in all events that lead to tumor cell detachment, invasion of the basement membrane and surrounding stroma and colonization of new sites. Cancer cells must first detach from the primary tumor cleaving cell-surface E-cadherin and down regulating cell-cell adhesion mechanisms [13]. Moreover, tumor cells must detach from the ECM and neighboring cells and the MMP-dependent cleavage of cell-matrix adhesion receptors can favor this process [14, 16]. It is well known that cancerous cells have the capacity of resisting and escaping from immune surveillance and it is becoming evident that MMPs are involved in these evasion mechanisms [19]. MMPs can

also contribute to reduce or increase infiltration and migration of leukocytes as they can cleave several chemokines (e.g. CXC 1, 4-7 and 12), in some cases, enhancing their activity, while in other cases, reducing it [20]. The establishment of tumor cells in new distant sites necessitates a strong interaction between the malignant cells and the host tissue stroma. Even though tumor cells express their own pattern/set of MMPs, it is becoming evident that they can direct MMP expression by endothelial cells, fibroblasts, and also leukocytes [8]. The MMPs secreted by the stroma are important in tumor-directed tissue remodeling, not only through physical structural modifications, but also and more importantly, through the release and increased bio-availability of molecules that can enhance tumor growth, angiogenesis and tumor cell migration [13]. The mechanisms of MMP expression are very complex. MMP transcription can be regulated by growth factors, cytokines, and oncogene products, which can be released by the stroma or by tumor cells themselves [5].

MMPs are overexpressed in many types of cancerous tissue and often it is not the cancer cells themselves, but rather the host stroma that produces the MMPs [21], suggesting that the tumor actively interacts and communicates with its surrounding stroma, causing overexpression of numerous MMPs. These MMPs assist in tumor invasion by remodeling of the surrounding matrix, and in promotion of tumor growth in a network where a single MMP cleaves certain matrix components and activates other latent MMPs. Distinct MMPs are active during different stages of tumor development [13]. When considering prognostic implications of MMP expression, the clinician should be aware that the host response to the tumor itself is able to provide important prognostic information. MMPs expressed by the tumor itself are also important for studying prognostic significance, as MMP expression is increased and strongly correlated with tumor invasiveness and poor prognosis reinforcing the concept that MMPs contribute to human cancer development [4, 22].

In some cases, mouse models of tumor progression have complemented some of the data from human tumors regarding the role of MMPs in tumor progression. In fact, in experimental models of metastasis, the injection of tumor cells into the tail vein of mice deficient in MMPs resulted in fewer tumors growing in the animals relative to mice that were wild type for MMPs, complementing data showing decreased prognosis in cancer patients with elevated MMPs. Studies provide further evidence for the use of specific MMPs as prognostic factors [3, 23].

Research to identify MMPs as potential new biomarkers has shown that MMPs are very specific for types and stages of cancer [13]. In fact, MMP-2, or Gelatinase A, one of the first cancer-associated MMPs discovered, was originally shown to selectively cleave type IV collagen [24], but numerous studies showed the prognostic significance of MMPs [4] in both overall survival and disease-free survival with several types of cancer [13].

MMPs can be useful for predicting tumor recurrence and metastatic risk [23]. In this respect, MMP determination from patient blood and urine has shown to have predictive value in estimation of hematogenous metastatic risk (e.g. in

lung and renal cell carcinoma) [7] demonstrating in an extremely wide number of prognostic studies, that increased expression and activity of MMPs negatively correlates with prognosis, thus indicating that these proteinases are strongly associated with a negative clinical outcome [13, 23].

Further complicating the story of MMPs as biomarkers is the data that show that not all MMPs are markers of poor prognosis and that sometimes, an augmented expression of MMPs correlates with better clinical results or treatment response. Worth noting are the apparently beneficial affects of MMP-2 in Hodgkin's lymphoma [25] and the positive correlation between the positivity of bone marrow blast cells for MMP-2 and prognosis [26]. In other neoplasms, there is evidence of a possible positive effect of MMPs (e.g. in melanoma and early breast cancer) [27, 28]. These data point to the necessity of further careful studies in order to target detrimental MMPs without altering those that may actually provide better outcome to cancer patients [29].

INHIBITION OF MMPs IN CANCER THERAPY

In order to inhibit growth and invasion of cancer cells, many hypotheses have been taken into consideration to block MMP activity in the extracellular environment [30]. Studies using small molecule inhibitors of MMPs in early disease provide evidence that MMP inhibitors (MMPIs) would be useful as therapies to treat and prevent metastasis [31]. In this way, there may be the possibility for a new approach to cancer treatment in addition to traditional cytotoxic therapy.

Several generations of synthetic MMPIs were tested in phase III trials in humans, which include synthetic collagen peptidomimetic MMP inhibitors, synthetic collagen non-peptidic MMP inhibitors, synthetic tetracycline derivatives and bisphosphonate inhibitors [5]. The results of these clinical trials have been disappointing as no therapeutic efficacy was seen [31]. Several reasons for the failure of MMPIs in human clinical trials have come to light. Firstly, the fact that many analyses have been performed in advanced stage tumors when malignant cells have already undergone metastatic transformation, while MMPIs may be more important in early stages of cancer due to their cytostatic rather than cytotoxic effects [32]. Another possible reason of failure in clinical studies is the lack of specificity of the inhibitors used, which can lead to unexpected and unwanted effects, since in some instances, MMP inhibition may be detrimental rather than therapeutic [5]. Moreover, a factor that strongly influences MMPI administration and consequent therapeutic efficacy is the high toxicity of almost all MMPIs, which cause, musculoskeletal syndrome that even though reversible, leads to treatment suspension in many cases [33]. Despite the large number of studies that catalog over-expression of MMPs in human cancers [3, 9, 13], additional work needs to be undertaken to better understand the influence and importance of specific MMPs on certain types and stages of cancer in order to use MMPIs effectively [5].

NATURAL MMP INHIBITORS

The negative results obtained in human clinical trial analyzing synthetic MMPIs [reviewed in 5] stirred up the impellent need for compounds that could be more effective in cancer treatment and this search found its answer in the field of natural compounds (Table 1). Recent studies have

taken into consideration compounds extracted from “*shark cartilage*” [34]. As MMPs are strongly linked to angiogenic and metastatic processes, the results of oral administration of investigational drugs (standardized extracts derived from shark cartilage, based on *Squalamine*, 3-N-1-[N-[3-(4-aminobutyl)]-1,3-diaminopropane]-7-cholestane 24-sulfate) Fig. (2A) (e.g. Neovastat, AE-941, U-995 etc.) were analyzed in regard to its anti-angiogenic and anti-metastatic effects on the activity of several MMPs [35]. It was demonstrated that Neovastat inhibits enzymatic activity of MMP-2 with minor inhibition of MMP-1, -7, -9 and -13. It is interesting to underline that western blot analysis evidenced the presence of TIMP-like proteins within AE-941 that could thus be responsible for its specific MMP inhibitory property [36]. Worthy of notice is that tissue inhibitors of metalloproteinase 1, 2 and 3 (TIMP-1, -2, -3) and tumor suppressor protein genes have been cloned and characterized from shark cartilage extracts [34, 37]. Neovastat exerts anti-angiogenic activity and this depends not only on its inhibition of matrix metalloproteinases, but also on the inhibition of the vascular endothelial growth factor (VEGF) binding to endothelial cells, VEGF-dependent tyrosine kinase phosphorylation, and VEGF-induced vascular permeability in mice. These effects and the absence of dose-dependent side effects of Neovastat makes this compound an ideal tool in cancer treatment, and several phase III clinical trials are currently evaluating the efficacy of Neovastat in patients with unresectable non-small cell lung cancer in addition to chemotherapy/radiotherapy and as a monotherapy in metastatic renal cell carcinoma patients [38].

Neovastat is in phase II trials in patients suffering from multiple myeloma [39] as well as in nonsmall cell lung cancer [40] and in renal cell carcinoma [41]. Neovastat is also being analyzed in the treatment of patients with advanced refractory cancer which demonstrated significant survival advantage and good tolerability [42]. All studies evidenced that Neovastat can interfere with several biochemical steps of the angiogenic pathway as it inhibits matrix metalloproteinase activity, blocks VEGF signaling, stimulates angiostatin production and inhibits endothelial cell proliferation [36, 42]. Neovastat probably exerts its endothelial-specific effects through the induction of apoptosis, as incubation of endothelial cells with this compound leads to chromatin condensation and DNA fragmentation [37]. These results are due to the activation of at least three key members of the caspase family and are specific for endothelial cells, as Neovastat did not induce detectable apoptosis in many other cell types. The mechanism is not yet clear by which Neovastat induces the caspase activity, but the presence of an endothelial-specific proapoptotic factor contributes to the anti-angiogenic properties of this compound [38]. The safety of Neovastat has been tested on a large number of patients (in some cases for more than 4 years). Its excellent safety profile and oral administration make Neovastat ideal for long-term treatments, alone or in combination with conventional therapies [42].

“*Genistein*” (4*H*-1-benzopyran-4-one,5,7-dihydroxy-3-(4-hydroxyphenyl)- Fig. (2B) is an isoflavonoid of the Leguminosae family, an intermediate in the synthesis of

other isoflavonoids that act as anti-microbial compounds protecting the plant from microbial invasion [43]. Isoflavonoids (such as genistein) are mainly found in soy products. It can be found in very high levels in powdered soy-bean chips and in a minor concentration in soy protein. The foods that are more rich in genistein are tofu, followed by miso and soy source. People following a soy-rich diet show very high levels of ingested isoflavones compared to people eating little soy products. Genistein possesses many potential beneficial effects towards prevention of cancer and cardiovascular disease. In fact, an interesting characteristic of genistein is that it is structurally similar to estradiol conferring estrogenic and anti-estrogenic properties as it can bind estrogen receptors and sex hormone binding proteins. Moreover, genistein shares structural similarity with tamoxifen, an anti-estrogen studied for the treatment and prevention of women with high risk of breast cancer [44]. Epidemiological studies of Chinese women involving healthy controls and subjects with breast cancer evidenced that soy consumption was strongly linked to reduced cancer risk, demonstrating that isoflavonoids found in soy products help protect from cancer risk [45]. Studies on animal models showed that when administered neonatally, genistein protects rats from chemically-induced mammary tumors by increasing latency, reducing tumor incidence and multiplicity and by causing a more rapid maturation of undifferentiated end buds to differentiated breast lobules [46]. This early mammary gland differentiation reduces epidermal growth factor signalling pathway in adulthood that in turn, suppresses development of mammary cancer [44]. Since genistein has been suggested to be responsible for the low rate of breast incidence in Asian women, it has been studied on breast carcinoma cells and it has been demonstrated that it inhibits cell proliferation of these cells at a specific G2/M arrest point and that it causes apoptosis [43].

Genistein also inhibits invasion in human breast carcinoma cell lines by diminishing the expression of MMP-2, -9, MT1-, MT2-, MT3-MMPs and increasing the level of TIMP-1 [47, 48]. In particular, the downregulation of MMP-9 was transcriptionally regulated at activation protein-1 sites in the MMP-9 promoter [49]. The effects of genistein in inhibiting MMP-9 expression and increasing TIMP-1 level was also studied *in vivo* in mouse xenografts, which showed that genistein blocked tumor growth and development and stimulated apoptosis. In addition, in a type of animal xenograft, genistein caused a decrease in vessel density and a reduction in the levels of VEGF and of transforming growth factor- β , thus inhibiting new blood vessel formation [49]. These studies shed light on several actions that genistein exerts, evidencing that it is not only involved in anti-proliferation processes, but that it may be useful in blocking a wide number of stages in breast cancer progression such as invasion and metastasis. Moreover, these results underline that genistein may be useful in prevention of cancer cell metastasis, since it transcriptionally regulates genes involved in tumor development and inhibits cancer cell invasiveness. Genistein, however, has been shown to be effective in inhibiting cell growth also in other types of cancer, such as stomach, bladder, lung and blood [43]. For stomach tumors, it is possible that genistein inhibits the growth of cancer cells as it triggers a signal cascade that leads to apoptosis [50],

Table 1. Main Natural Compounds with Effective MMP Inhibition Activity (Nutraceutical/Biodrugs)

Source	Compound (PATENT number)*	Inhibition of MMP Forms	References
<i>Shark cartilage</i>	<i>Neovastat</i> ® (AE-941), <i>Squalamine</i> , U-995 (US2001001041)[35]	MMP-1, -2, -7, -9, -13	[34-37]
<i>Soybean seeds</i> <i>Genista Tridentata</i> L.	<i>Genistein</i>	MMP-2,-9, MT1-, MT2-, MT3MMP MMP-9 MMP-2, MMP-9 MMP-2	[47, 48], [49, 51, 54] [52] [53]
<i>Citrus fruit</i> <i>Aurantii fructus immaturus</i>	<i>Nobiletin</i> (JP2000080035) [55]	MMP-2, MMP-9 MMP-7 MMP-9 MMP-1, MMP-9	[56] [57] [58] [59]
<i>Berry fruits</i> <i>Myrica nagi</i> Thieb.	<i>Myricetin</i>	MMP-2	[60]
<i>Curcuma rhizome</i> <i>Curcuma longa</i>	<i>Curcumin</i> (JP2001139466)[63] <i>Xanthorhizzol</i>	MMP-2, MT1-MMP MMP-9 MMP-2 MMP-2, MMP-14 MMP-9	[68] [69] [70] [71] [75]
<i>Green tea leaves</i>	<i>Catechins</i>	MMP-2, MT1-MMP MMP-2, MMP-9 MMP-2, MMP-9, MMP-12	[86] [89, 97] [90, 91]
<i>Cocos nucifera</i>	(JP2000226329)[80]		
<i>Black tea leaves</i> <i>Camellia sinensis</i>	<i>Theaflavins</i>	MMP-2, -9	[96]
<i>Grapes</i> <i>Veratrum grandiflorum</i>	<i>Resveratrol</i>	MMP-9	[100, 101]
<i>Cinnamomum</i> , <i>Magnolia</i> and <i>Euonymus</i>	<i>Unknowns</i>	MMP-9	[102]

*: Indicative not exhaustive patent data supplied from worldwide database (<http://v3.espacenet.com>).

while in animal studies on salivary adenoid cystic carcinoma it was evidenced that genistein significantly reduced the number of metastases in injected mice and it also augmented the apoptosis index. Moreover, it caused a reduction in MMP-9 and VEGF expression exerting moderate inhibitory effects on metastasis of adenoid cystic carcinoma [51]. Genistein further demonstrated its anti-invasion and anti-metastasis properties in human sarcoma cells, as it diminished the conversion of the latent form of MMP-2 and -9 to their respective active forms and strongly increased the mRNA levels of TIMPs, thus turning upside-down the MMP/TIMP balance and leading to matrix degradation inhibition and to blockage of cell invasion [52]. Recent studies indicate that consumption of soy is correlated to low metastatic prostate cancer incidence and so genistein has been analyzed in this pathological situation. Cell invasion and metastatic process have been taken into consideration and it was found that genistein regulates these biological mechanisms by inhibiting MMP-2 activity and blocking MMP-2 induction by TGF- β in prostate cell lines [53]. Genistein inhibits MMP-2 induction and cell invasion as it

blocks the activation of p38 mitogen-activated protein kinase (MAPK) by TGF- β . *In vivo* studies on prostate cancer bone metastasis show that genistein also inhibits tumor growth and invasion by downregulating the expression of MMP-9 [54]. It is worthwhile noting that genistein exerted its inhibition on cell invasion at very low concentration levels, which equal those achieved with dietary assumption of soy products [53].

Among the natural compounds that help to protect from pathological states such as cancer, other flavonoids have been analyzed including the citrus flavonoid “*Nobiletin*” (4*H*-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-) Fig. (2C), which possesses important anti-cancer characteristics [55]. In particular, in *in vitro* studies analyzing the effects of various flavonoids on brain tumor cells, nobiletin strongly inhibited invasion, migration and cell adhesion in addition to down-regulating the secretion and activity of MMP-2 and -9 contributing to generate a less invasive cell phenotype [56]. Nobiletin may be effective in other cancer types such as human colorectal cancer and gastric carcinoma, as *in vitro* studies on human colorectal

cells treated with this flavonoid showed decreased expression of proMMP-7 and of its mRNA, which was dependent on the reduction of activator protein-1 DNA binding activity [57]. Nobiletin also inhibited proliferation of cancer cell and expression of MMP-9 in studies on human gastric carcinoma in animal models [58]. It has been demonstrated that nobiletin exerts its anti-invasive properties not only by downregulating the expression of certain MMPs, but also by increasing the level of TIMPs. In fact, nobiletin reduced the production of pro-MMP-1 and -9 and caused an increase in the production of TIMP-1 in human fibrosarcoma cell lines [59]. All these results evidencing the positive anti-tumor effects of nobiletin make it a favorable candidate for therapeutic treatment of tumor pathologies. Flavonoids seem to be strongly involved in inhibiting metastatic processes as myricetin, a flavonoid found in berries, fruits, vegetables, herbs and tea, greatly decreased the activity of MMP-2 in human colorectal carcinoma cell lines. "Myricetin" (3,5,7-trihydroxy-2-(3,4,5-trihydroxy-phenyl)-4H-1-benzopyran-4-one) Fig. (2D) may inhibit MMP expression and activity by blocking the cascade that brings to c-Jun expression [60].

One of the strongest natural chemopreventive compounds is "Curcumin" (1E,6E-1,7-bis(4-hydroxy-3methoxyphenyl)hepta-1,6-diene-3,5-dione) Fig. (2E). It derives from *Curcuma* species that are medical plants in Indonesia and curcumin is a major yellow pigment extracted from the ground rhizoma of these plants. Three curcuminoids named curcumin, demethoxycurcumin and bis-demethoxycurcumin are mostly found in curcuma plants and their concentration varies according to the site and cultivation period, and it seems that *Curcuma longa* has the highest concentration of curcumin [61]. Curcumin, other than possessing potent antioxidant and anti-inflammatory effects, blocks tumor invasion and development. It appears to suppress the onset of tumors as well as their growth and metastasis in many cellular systems and animal models [62, 63]. Curcumin does not cause dose-limiting toxicity and can be taken orally. It is, therefore, an ideal candidate in the prevention and treatment of cancer and thus has the potential characteristics for a cancer chemopreventive agent. Curcumin inhibits several types of cancer, such as adenocarcinoma, forestomach, duodenal and colon cancer and it also prevents metastasis formation in animal models [64-67]. Moreover, curcumin reduces the risk of lung cancer associated with smoking, showing that curcumin strongly reduced its carcinogenic effect [68]. Owing to the many positive results curcumin showed in fighting cancer, it has undergone several studies to better understand its way of action. A report on murine melanoma cells evidenced that a treatment with curcumin reduced the activity of MMP-2 and MT1-MMP and that this inhibition remained for several days' post-treatment suggesting the hypothesis that the decrease in MMP level may reduce metastatic risk [69]. In support to the assumption that curcumin blocks malignant cell invasion, thus having anti-metastatic effect, *in vitro* studies of human hepatocellular carcinoma showed that curcumin inhibited cellular migration and invasion by diminishing MMP-9 secretion in a dose dependent manner [70], while in human breast cancer cells the anti-invasive effects of curcumin depended on the decrease in MMP-2 and on the increase in TIMP-1 levels. Moreover, curcumin reduced the transcription of VEGF and

of basic fibroblast growth factor [71]. The anti-metastatic properties of curcumin were investigated on highly invasive lung adenocarcinoma cells and it inhibited the expression of MMP-14 and of MMP-2 (both in its pro-form and total active form). Being these two MMPs crucial for tumor growth, invasion and metastasis, curcumin may block malignant cells through the inhibition of these enzymes. Curcumin can regulate certain transcriptional factors and its inhibition of the AP-1 system may cause the downregulation of MMP-14 and the subsequent inhibition of MMP-2 [72]. In addition, it is interesting to note that in animal models, curcumin suppressed the activation of the transcription factor c-jun/AP-1, which is important in the signaling of induced tumor promotion, suggesting that curcumin may cause the inhibition of c-jun/AP-1-mediated gene expression [73]. It is clear that curcumin can regulate and control several steps that lead to tumor development; in fact, it has been demonstrated that tumor promotion induced by TPA is strongly inhibited by curcumin [74]. Curcumin exerts its action at DNA level, on the messenger level and on the enzyme level leading to the suppression of components essential for cell proliferation. It seems that curcumin can keep the cell in its normal functional state by attenuating or blocking the activity of such nuclear components that may otherwise lead to uncontrolled proliferation and tumor growth [75].

"Xanthorrhizol", is a sesquiterpenoid compound isolated from the rhizome of *Curcuma*, and is widely used for the treatment of various pathological states. Xanthorrhizol has been analyzed in an animal model of lung metastasis and it inhibited tumor growth and the metastatic activity of cancer cells. It is probable that xanthorrhizol may exert its anti-metastatic properties by downregulating MMP expression [76]. At the genetical level, xanthorrhizol attenuated the expression of the phosphorylated ERK, and since ERK signaling pathway is involved in metastatic processes and in the production of MMPs [77], it is possible that this could be the mechanism responsible for the suppression of cell metastasis [76].

In Chinese medicine, tea is a fundamental element and it is considered one of the most potent substances that can help maintain health and prolong life, and these health benefits have been scientifically established and are related to the presence of polyphenols called "flavonoids". Tea (*Camellia sinensis*) is available in three forms: black, green and oolong, which differ in the type of flavonoids they contain. Green tea has more simple flavonoids called "catechins", while the brewing of black tea oxidizes the catechins, destroying their beneficial effect and transforming them into complex flavonoids called theaflavins and thearubigins [78]. Recent studies suggest that the consumption of tea, and green tea in particular, may help prevent cancer [79] and evidence that it has anti-mutagenic and anti-carcinogenic properties as its increased administration to breast cancer patients decreased the number of axillary node metastases [80]. Green tea diminished the incidence of stomach and rectal cancer and of mouse skin tumors induced by UV-B radiation [81, 82]. Even though the beneficial effects of tea have been attributed to the anti-oxidative effect of its polyphenols, their exact mechanism of action is not yet completely understood and many studies have been undertaken to analyze the effects of

the various tea catechins: (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) Fig. (2F). A way by which EGCG may decrease tumor size is through the binding to urokinase, which is a proteolytic enzyme, often overexpressed in human cancer, necessary for invasion and metastasis of cancer cells. In this way, EGCG interferes with the recognition of urokinase to its substrates, inhibiting its activity [83]. Among all the catechins present in green tea, EGCG seems to be the most effective in cancer chemoprevention. Studies on hamster cells showed that EGCG protects normal cells against genotoxic effects as it protected the cells from the cytogenetic changes that would have otherwise been induced by the toxic agent, and moreover, EGCG eliminated mutated cancer cells through the induction of apoptosis, probably by the activation of the caspase cascade [84]. The beneficial effects of EGCG were tested on chronic lymphocytic leukemia B cells isolated from leukemic patients. This experiment was a big challenge, as these cells show a strong resistance to apoptosis as a result of their capacity for secreting and binding VEGF. EGCG greatly reduced VEGF-receptor phosphorylation, blocking the VEGF-dependent signaling that protects cancer cells from apoptosis. Of great importance, is the fact that catechin can exert its preventive activity at low concentrations that can be achieved with no risk for side effects [85]. The inhibitory activity of EGCG towards VEGF can also interfere with the initial stages of tumor development, because tumor cells need to be provided with new blood vessels, and the inhibition of VEGF signaling may block angiogenesis and the growth and invasion of tumor cells in various types of cancer. It has been hypothesized that EGCG can also inhibit angiogenesis and tumor growth also through the inhibition of enzymes such as the MMPs, which are deeply involved in these events. Studies on human umbilical vein endothelial cells confirmed this hypothesis as EGCG greatly and specifically inhibited MT1-MMP, suppressing cell invasion, and it also decreased the presence of the active form of MMP-2 [86]. Moreover, by blocking angiogenesis, this catechin suppressed tumor growth in colon carcinoma and sarcoma-bearing mice [87]. A surprising and unexpected result was obtained from the study of human colorectal cancer cells treated with EGCG: in this case, polyphenol addition caused an increase in both intracellular and extracellular pro-MMP-7 protein levels in a dose- and time-dependent manner. In particular, EGCG increased the mRNA levels of this MMP probably through the activation of c-jun and c-FOS pathway, leading to AP-1 activation [88]. However, it is interesting to note that also ECG induced MMP-7 expression, while EC and EGC did not. Moreover, EGCG down-regulated the expression of the two gelatinases MMP-2 and -9 and it also increased the expression of TIMP-1 and TIMP-2, which block the activity of activated MMPs [89]. This strong inhibitory activity of tea polyphenols towards gelatinases was further investigated in cell cultures of human brain tumors such as glioblastoma and pituitary tumors which are known to have high levels of MMP-2 and -9. The results confirmed that EGCG inhibits MMP-2 and -9, and also MMP-12, and it seems probable that EGCG interacts with the MMPs through its gallate residues. In this study, the levels of proMMP-2 were unaffected by EGCG,

suggesting that the inhibition of this gelatinase acts at the activation level, while for MMP-12, a specific interaction between EGCG and the catalytic site of the enzyme may be the cause of MMP inhibition [90]. An intriguing study showed that EGCG forms a reversible complex with MMP-2, thus inhibiting enzymatic activity. In particular, EGCG not interfere with the binding of MMP-2 to native or denature-type I collagen, but it increased the binding of both pro and active for of MMP-2 to TIMP-2 [91].

It is important to note that the effects of EGCG are obtained at concentrations that are physiologically achieved representing an interesting anti-angiogenic tool. Animal models of a wide number of cancers (skin, liver, mammary gland, stomach, pancreas, prostate), collectively showed that tea treatment inhibits tumor multiplicity and tumor incidence and suppresses distal metastasis formation. Tea components may block tumorigenesis at one of its several stages and they may also increase the apoptotic index of tumors while decreasing the proliferating index and microvessel density [92]. In particular, EGCG inhibited lung tumors in mice previously treated with a strong carcinogenic agent formed during tobacco smoking. Tea polyphenols may modulate cell growth by arresting the cell cycle or inducing apoptosis through the increase in p53 levels thus resulting in strong anti-proliferative and anti-apoptotic agents on various cell types. It has been demonstrated that these compounds inhibit AP-1 activity, which regulates a wide range of biological events and plays a role in the apoptotic phenomenon [93]. EGCG could mediate its activity through the selective blocking of growth factor receptor-mediated signal transduction, either by competition with ligand or inhibition of kinase activity [94].

Although mayor attention has been concentrated on catechins, also caffeine seems to be important in the inhibition of carcinogenesis, as it has been shown that decaffeinated teas are much less effective in reducing the incidence and multiplicity of UVB-induced skin tumors in animals, and addition of caffeine restores the activity of decaffeinated teas [92]. Among green tea polyphenols, EGC can inhibit tumor cell proliferation. This catechin inhibits DNA replication in leukemia cell lines, resulting in an accumulation of S phase cells and inhibition of the S-G2 progression evidencing a possible mechanism for the suppression of proliferation of tumor cells [95].

Black tea also exerts potential useful effects. *Theaflavin* Fig. (2G) and theaflavin digallate, which are components of black tea, inhibited tumor cell invasion and also the expression of MMP-2 and -9, suggesting that tumor invasion may be due to the inhibition of gelatinases [96].

But polyphenol are not restricted only to tea. Catechins extracted from *Cocos nucifera* inhibited cell proliferation in a leukemic cell line and on normal human peripheral blood lymphocytes showing a dose-dependent effect [97].

“Resveratrol” (1,3-benzenediol, 5-(2-(4-hydroxyphenyl) ethenyl)-, (*E*)-; 3,5,4'-trihydroxy-trans-stilbene; (*E*)-5-[2-(4-hydroxyphenyl)ethenyl]-1,3-benzendiol) Fig. (2H) is a natural phytoalexin found in grapes and other plants [98]. Although resveratrol has been demonstrated to have such

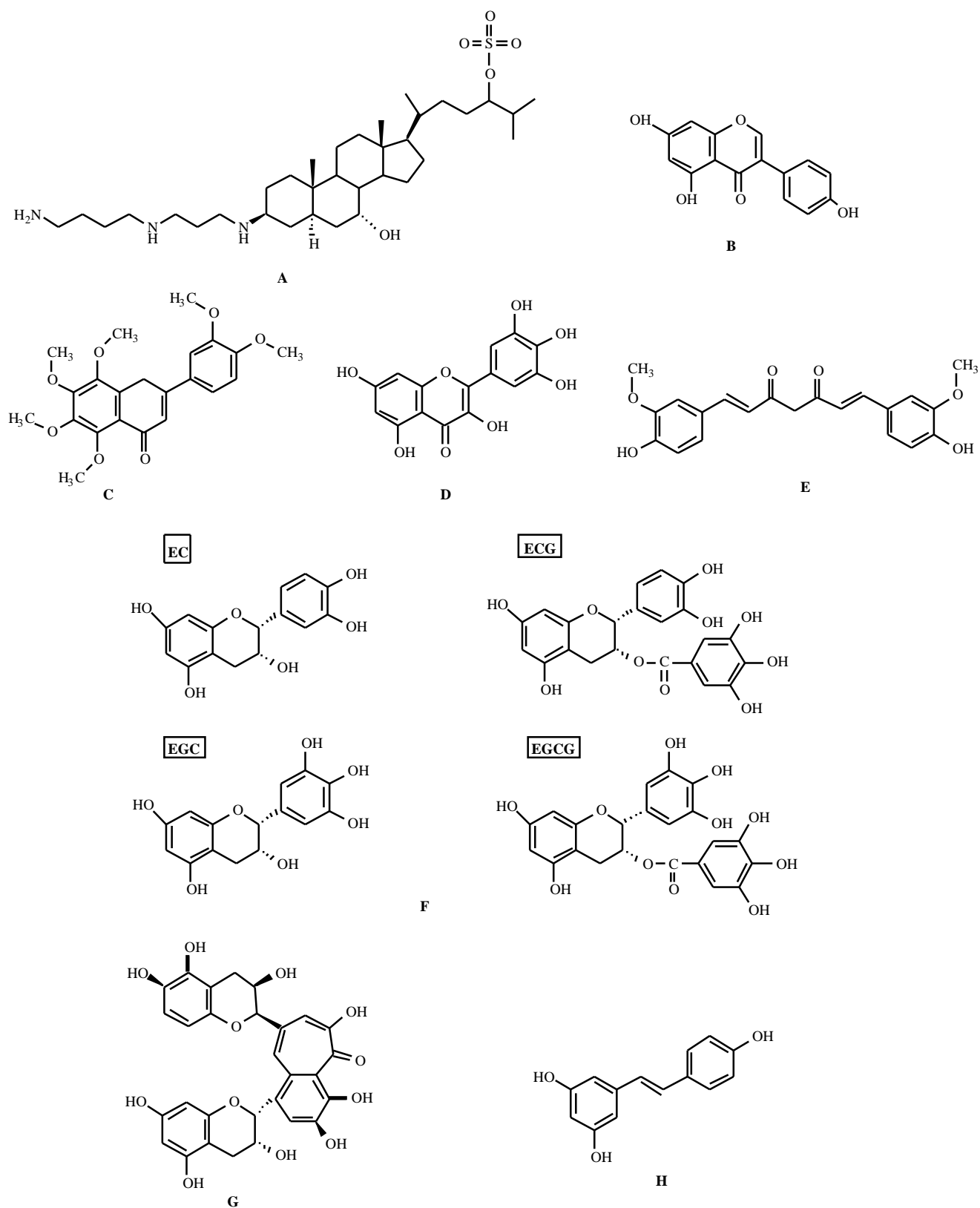


Fig. (2). Chemical structure of natural bio-drugs with matrix metalloproteinase inhibitory effect.

A, Squalamine. B, Genistein. C, Nobiletin. D, Myricetin. E, Curcumin. F, Catechins. G, Theaflavin. H, Resveratrol.

extensive pharmacological properties [99], recently it was found to have direct inhibitory effect on MMP enzyme activity, so modulation of resveratrol on MMP gene expression may be one of its anti-inflammatory mechanisms and anti-tumor activity [100, 101]. These activities may have a therapeutic potential given a novel means of controlling growth and invasiveness of tumors [99].

A study on extracts of oriental medicine herbs was analyzed for their inhibitory activity towards MMP-9 and a wide number of plants showed an inhibitory activity on the gelatinase. In particular, fractions from *Cinnamomum cassia*, *Magnolia obovata*, *Magnolia officinalis*, and *Euonymus alatus* had a very strong inhibitory effect [102].

CURRENT AND FUTURE DEVELOPMENTS

Matrix metalloproteinases are considered promising targets for cancer therapy due to their strong involvement in malignant pathologies, as their expression is upregulated in such diseases and because they can degrade all components of the extracellular matrix. Preclinical studies analyzing MMP inhibition in tumor models brought positive results rising the idea that the development of strategies to inhibit MMPs may be a powerful tool to help defy cancer. Experimental models have been carried out to study the blocking of MMP gene transcription based on targeting extracellular factors, signal-transduction pathways or nuclear factors that activate expression of these genes.

The importance of nutraceuticals in cancer prevention and treatment remains largely under-exploited, despite increasing evidence showing that these molecules have both chemo-preventive and chemo-therapeutic ability. Notwithstanding, the considerable progress made in the design of novel anti-cancer drugs in recent years, one clear lesson from the recent decades of research into cancer is that, although we can treat cancer and induce remission, survival rates have changed little in most cancers [2]. Moreover, most anti-cancer drugs have several toxic side-effects that may produce a poor quality of life for cancer patients and considerable cost in supporting care [32].

From this point of view, one could conclude that the chances of developing effective anti-MMP therapies would greatly increase with improved knowledge of the contribution of MMPs to the progression of specific cancer types and stages with the appropriate tools for evaluating MMP inhibitory activity at both the molecular and clinical levels. Although, at the moment, the future of MMP inhibitors in the treatment of cancer appears to lie primarily in the "hands" of the pharmaceutical companies, what remains clear is that there is still much basic and translational groundwork to be done to develop and validate tools identifying tumor expressing target enzymes and, primarily, to assess the efficacy of specific compounds (and optimal doses) that significantly limit tumor-associated proteolytic activity.

Whether MMP inhibitors will become a standard in the cancer armament is unclear [5], but clinical research needs to focus on improving the design of trials that better assess natural agents with tumoricidal activity. Nutraceuticals or natural biodrugs may offer several advantages as anti-cancer products because these diet-derived compounds are non-

toxic, widely available and inexpensive. Their activity appears to act through multiple targets and suggesting the presence of several natural components that could synergistically act together to control tumor growth and neovascularization. It is clear that anti-cancer biodrugs designed by nature and used for several thousands of years with little toxicity may prove useful in both treating and preventing cancer. Pleiotropic multiple mechanisms of inhibition of matrix metalloproteinases by biodrugs may be of significant importance to our understanding of the mechanism by which nutraceuticals elicit their anti-angiogenic, anti-tumoral and anti-metastatic effects. The strong inhibition of gelatinolytic activities, the control of MMP gene expression and the antagonization of MMP activation by nutraceuticals may provide novel and plausible molecular mechanisms for how biodrugs obtained from natural dietary constituents may inhibit the growth and vascularization of rapidly proliferating neoplastic cells, through the additional support of substances created by Nature, with specific MMP inhibition activity useful in cytostatic therapies of the innovative treatment of cancer.

ABBREVIATIONS

MMP	=	matrix metalloproteinase
ECM	=	Extracellular matrix
MT-MMP	=	Membrane-type MMP
RECK	=	Reversion-inducing cysteine-rich protein with kazal motif
TIMP	=	Tissue inhibitors of MMPs
IGF-BP	=	Insulin-like growth factor binding protein
IGF	=	Insulin-like growth factor
TGF-	=	Transforming growth factor
ICAM-1	=	Intercellular adhesion molecule-1
TNF	=	Tumor necrosis factor
Fas L	=	Fas ligand
MCP-3	=	Monocyte chemoattractant protein-3
MMPI	=	Matrix metalloproteinase inhibitor
VEGF	=	Vascular endothelial growth factor
MAPK	=	Mitogen-activated protein kinase
ERK	=	Extracellular signal-regulated kinase
C (+)	=	Catechin
EC (-)	=	Epicatechin
EGC (-)-	=	Epicatechin gallate
EGCG (-)-	=	Epigallocatechin gallate

*Medicus curat, natura sanat . "Doctor cures, nature saves"
(Regimen Sanitatis Salernitanum,1607)*

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