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# Biological action of two tomato cysteine-rich miniproteins

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# INTRODUCTION

## NATURAL COMPOUNDS AND PHARMACOLOGY

Around 80% of the world population, mainly in the non-developed countries, has the first option for medication in Primary Health Care in natural products (Mahady, 2001). Natural products, as disease remedies, have a history of nearby 5000 years (India, China and Greece), whereas isolated pure chemicals or synthetic drugs have been around for 100 years, since Bayer synthesized acetylsalicylic acid (aspirin) at the end of XIX century. Nowadays, around half of the drugs currently in clinical use are of natural product origin (Butler, 2005; Newman *et al.*, 2003) and nutraceuticals, i.e. 'natural' substances isolated or purified from food and used in a medicinal fashion, have appeared on the market. These nutraceuticals are pharmaceutical preparations (pills, powders, capsules, vials, etc) containing food bioactive compounds as active principles. Scientific research has supported the biological activity of many of these food phytochemicals; in fact several naturally derived food substances have been studied, showing an extensive spectrum of biological activities such as stimulation of the immune system, anti-bacterial, anti-viral, anti-hepatotoxic, anti-ulcer, anti-inflammatory, anti-oxidant, anti-mutagenic, and above all anti-cancer effects (Miyata, 2007; Espín *et al.*, 2007). Cancer is mostly a preventable disease (Glade, 1999). One of the most important ways to reduce cancer risk is the habitual consumption of foods containing bioactive compounds that protect against cancer. Several studies have documented the relationship between decreased cancer risk and high consumption of vegetables, including cabbage, cauliflower, broccoli, brussels sprout, tomatoes, and fruits such as, apples, grapes, and berries (Vainio and Weiderpass, 2006; Gordaliza, 2007). Furthermore, a variety of grains, cereals, nuts, soy products, olives, beverages such as tea and coffee, and spices including turmeric, garlic, ginger, black pepper, cumin and caraway confer a protective effect against cancer (Lila, 2007; Williams and Hord, 2005). Vitamin E, selenium, vitamin D, green tea, soy, and lycopene have all been examined in human studies and in general, it is possible to say that natural products consist of a wide variety of biologically active phytochemicals including phenolics, flavonoids,

carotenoids, alkaloids and nitrogen containing as well as organosulfur compounds, which have been shown to alter carcinogenesis (Table 1) (Nishino *et al.*, 2007).

**Table 1.** Source, mechanism of action, molecular targets, and cancer type target of some of the more promising antitumoral natural compounds (adapted from Amin *et al.*, 2009).

| Agent | Natural Source | Mechanism of Action | Molecular Targets | Cancer Type |
|---|---|---|---|---|
| Green tea (polyphenols and EGCG) | *Camellia sinensis* (green tea) | Antioxidant, anti-mutagenesis, anti-proliferation (cell cycle arrest, apoptosis), anti-inflammation, anti-angiogenesis, immuno-modulator | p53, p73, p21, Bax, EGFR, AKT, NF-*k*B, Bcl-2, cyclin D1, COX-2, VEGF, MMP-2/9, STAT3, ERK1/2, AP-1, IL-12, $CD8^+$ T-cell | Lung, skin, ovarian, prostate, solid tumors, cervica, breast, bladder, leukemia, lymphoma, osophageal |
| Curcumin | *Curcuma longa* (turmeric powder) | Antioxidant, anti-proliferation (cell cycle arrest, apoptosis), anti-inflammation, anti-angiogenesis, immunomodulator | EGFR, IGF-1R, AKT, NF-*k*B, Bcl-2, COX-2, ERK, AP-1, Sp, VEGF, VEGFR1, MMP-2/9, p53, p21, Bax, STAT3/5 | Multiple myeloma, rectal, colon, pancreatic, osteosarcoma, colorectal |
| Luteolin | Artichoke, broccoli, celery, cabbage, spinach, green pepper, pomegranate leaves, peppermint, tamarind, and cauliflower | anti-inflammation, anti-allergy, anti-proliferation (G1 and G2/M arrest, apoptosis), antioxidant, pro-oxidant | JNK, p53, DR5, BAX, p21, PUMA, EGFR, IGF-1R, AKT, NF-*k*B, Bcl-2, CDK, ERK, STAT3 | |
| Resveratrol | Red wine, grapes (mainly in the skin), mulberries, peanuts, vines, pines | Antioxidant, anti-proliferation (cell cycle arrest and apoptosis), anti-angiogenesis, anti-inflammation | SOD, catalase, glutathione, 1glutathione, AKT, NF-*k*B, iNOS, COX-2, STAT3, survivin, p53, p21, BAX, BAK, DR | Colon, solid tumors, colorectal, colon, follicular lymphoma |
| Genistein | Soybeans and soy products, red clover (*Trifolium pretense*), Sicilian pistachio *(Pistacia vera)* | Antioxidant, anti-proliferation (growth inhibition, cell cycle arrest, apoptosis), anti-angiogenesis, anti-inflammation | AKT, NF-*k*B, Bcl-2, survivin, cyclin D1, COX-2, MMP-2/9, p53, p21, GADD153, Bax, STAT3/5, ERK1/2, CDK1, AP-1, IGF-1R | Breast, prostate, kidney cancer, melanoma (skin), bladder, leukemia, lymphoma |
| Pomegranate | *Punica granatum* (pomegranate fruit, pomegranate juice, pomegranate seed and seed oil) | Antioxidant, anti-proliferation (growth inhibition, cell cycle disruption and apoptosis), anti-angiogenesis, anti-inflammation | NF-*k*B, Bcl-2, COX-2, VEGF, ERK, JNK, p38, AKT, mTOR, iNOS, cyclin, CDK, p21, p27, BAX, BAK | Prostate, BHP, follicular lymphoma |
| Lycopene | Tomatoes, guava, rosehip, watermelon, papaya, apricot and pink grapefruit; most abundant in red tomatoes and processed tomato products | Antioxidant, anti-proliferation (growth inhibition, cell cycle arrest, apoptosis), anti-angiogenesis, anti-inflammation, immunomodulator | Cyclin D1, Bcl-2, Bcl-xL, AKT, BAD, NF-*k*B, MMP-9, Sp-1, IGF-BP3 | Prostate, BPH, prostatic intraepithelial neoplasia, precancerous condition |
| Lupeol | Mango, olive, fig, strawberry, red grapes | Antioxidant, anti-mutagenesis, anti-inflammation, anti-proliferation (cell cycle arrest, apoptosis, induction of differentiation) | 14-3-3-$\sigma$, BAX, p21, Fas, Bcl-2, cyclin D1/2, Ras, NF-*k*B, COX-2, NOS, AKT | |
| n-3 polyunsaturated fatty acids | Corn oil, sunflower oil, safflower oil, and olive oil, soybeans, walnuts, dark green leafy vegetables such as kale, spinach, broccoli, and Brussels sprouts, and seeds or their oils such as flaxseed, mustard seed, and rapeseed (canola) | Anti-inflammation, apoptosis, cell cycle arrest, lipid peroxidation | NF-*k*B, Bcl-2, STAT3, p53, Bax, p21, Fas/FasL, PPAR-$\gamma$, RXR, Ras, ERK 1/2 | Prostate, breast, soft tissue, head and neck, colorectal, hepatocellular carcinoma, precancerous condition, colitis, mucositis, esophageal, follicular lymphoma, cholangiocarcinoma |
| Ginkolide B | *Ginko biloba* | Antioxidant, anti-angiogenic, apoptosis | PAFR, NO, iNOS, eNOS, JNK | |

Abbreviations: EGCG, epigallocatechin-3-gallate; BPH, benign prostate hyperplasia; EGFR, epidermal growth factor receptor; NF-*k*B, nuclear factor-*k*B; COX-2, cyclo-oxygenase-2; VEGF, vascular endothelial growth factor; MMP-2/9, matrix metalloproteinases; IL-12, interleukin 12; IGF-1R, insulin-like growth factor-1 receptor; Sp, stimulating protein; VEGFR1, vascular endothelial growth factor receptor 1; JNK, Jun-N-terminal kinase; CDK, cyclin-dependent kinase; ERK, extracellular signal–regulated kinase; SOD, superoxide dismutase; mTOR, mammalian target of rapanycin; iNOS, inducible nitric oxide synthase; DR, death receptor; IGF-BP3, insulin-like growth factor binding protein 3; PPAR-g, peroxisome proliferator-activated receptor-g; PAFR, platelet activating factor receptor; NO, nitric oxide; eNOS, endothelial nitric oxide synthase.

In this framework, cancer chemotherapy presents an ideal opportunity for natural product–inspired drug discovery and development. An ideal cancer chemopreventive agent should have little or no toxicity, high efficacy in multiple sites, capability of oral

consumption, known mechanisms of action, low cost, and human acceptance. Indeed, in recent years, natural products have received great attention for cancer prevention owing to their various health benefits, noticeable lack of toxicity and side effects, and the limitations of chemotherapeutic agents (Manson *et al.*, 2005).

Unfortunately, many of the most promising natural compounds are available only in extremely small quantities, especially those from marine organisms such as sponges. In the last few years, intense research efforts have been devoted to overcome the limits imposed by nature. There are two main targets of this research: large-scale production and the optimization of the intrinsic biological activity of a natural product. A successful example of improvement in production strategy is the microtubule-stabilizing agent discodermolide that is available only in minute amounts from its natural origin (a marine sponge). Through the continued evolution and optimization of synthesis strategies, discodermolide is now available in the desired amount (Paterson and Florence, 2003; Smith III *et al.*, 2000). Optimization of the intrinsic biological activity of a natural product implies the design and the preparation of natural product analogs that allow us to tailor and enhance the drug-like properties (bioactivity, pharmacokinetics, solubility, etc.) of the natural active principles. Not only structural modifications can lead to biologically effective drug candidates, but also permutations, including structural simplification with the removal of unneeded functional groups and stereochemistry, facilitating chemical synthesis. A fine example of a natural product-inspired drug candidate is the potent oncolytic (cancer cell-killing) agent E7389, currently in phase I clinical trials. E7389 arose from extensive studies aimed to remove unneeded functional groups from halichondrin B (Aicher *et al.*, 1992), a highly cytotoxic and complex marine natural product in order to simplify chemical synthesis. Another example is the design and development of a new class of anticancer drugs that mimic the Smac motif and act as antagonist of inhibitor of apoptosis proteins (Li *et al*., 2004; Sun *et al*., 2004).

Of course, the opportunities for the preparation of natural product analogs are not restricted to the discovery of anti-cancer drug candidates. For example, in the case of anti-infectives, analog design may allow us to circumvent drug resistance, in a manner that again cannot be matched by standard methods for antibiotic development (Charest *et al.*, 2005).

Why do natural products possess such extraordinary specificity and potency compared to artificially designed molecules? The answer lies in evolutionary selection, nature's own high-throughput screening process for the optimization of biologically active compounds. Natural products tend to possess well-defined three-dimensional structures, embellished with functional groups (providing hydrogen bond acceptor/donors, etc.), which have been fine-tuned into a precise spatial orientation. Additionally, the structures of the biological targets of such natural products (e.g., protein binding sites) are often well conserved among proteins of markedly different genetic sequences (Zhang and DeLisi, 1998; Anantharaman *et al.*, 2003), so much so that metabolites that have evolved for a certain purpose and mode of action by a producing organism may exert different, yet equally potent, effects in other settings.

In conclusion, the continued isolation of an increasing range of novel bioactive metabolites suggests that the plant kingdom represents an immense reservoir of potentially powerful therapeutic agents whose surface has been barely scratched.

## TOMATO AS PHYTOTHERAPIC

Tomato was introduced in human diet approximately five hundred years ago and during the last two centuries has become one of the most important horticultural crops due to the worldwide consumption of its fruit and products such as sauces, tomato paste, canned tomatoes and juices. Many tomato products are good sources of potassium, folate, and the vitamins A, C, and E, as demonstrated in Table 2 (USDA Nutrient Data Bank).

**Table 2.** Nutrient composition of tomatoes and related tomato products (from USDA Nutrient Data Bank)

| | Tomato products (*per 100 g*)[2] | | | | |
|---|---|---|---|---|---|
| Nutrient | Raw tomatoes | Catsup | Tomato juice | Tomato sauce | Tomato soup |
| Potassium, *mg* | 237 | 382 | 229 | 331 | 181 |
| $\alpha$-tocopherol, *mg* | 0.54 | 1.46 | 0.32 | 2.08 | 0.50 |
| Vitamin A, *IU* | 833 | 933 | 450 | 348 | 193 |
| Vitamin C, *mg* | 12.7 | 15.1 | 18.3 | 7.0 | 27.3 |
| Total folate, $\mu g$ | 15 | 15 | 20 | 9 | 7 |

[2] USDA Nutrient Data Bank numbers: raw tomatoes 11529; catsup 11935; tomato juice 11540; tomato sauce 11549; tomato soup 6359

In comparison with the other regularly consumed vegetables, only carrots are a better dietary source of vitamin A than tomato-based foods. In addition to their micronutrient benefits (vitamins, minerals, etc.), tomatoes also contain valuable phytochemicals, including carotenoids and polyphenols. For instance, carotenoids, such as the red pigmented lycopene, β-carotene, a pro-vitamin A, phytoene, and phytofluene are all found in abundance in raw tomatoes and tomato products (USDA Nutrient Data Bank; Tonucci *et al.*, 1995). Several studies have indicated that regular consumption of tomato fruit is consistently associated with lower risk of cardiovascular disease and several types of cancer, including prostate cancer (Etminan *et al.*, 2004; Hwang and Bowen, 2002). Rather, with prostate cancer, tomato appears the appropriate food choice that can slow the onset of the disease and that can play a role in prevention (Wilkinson and Chodak, 2003; Cohen, 2002). Although tomato phytochemicals are thought to contribute to the reduced risk of human diseases, the active principles of tomato fruit have eagerly been searched, but their identification is still incomplete. β-carotene and lycopene have been indicated as putative active principles, however evidence of their role as the main cardio-protective and/or anti-cancer principle is either negative or not conclusive (Canene-Adams *et al.*, 2007). In fact, evidence suggests that lycopene by itself might have some anti-prostate cancer activity or might be protective against lipid peroxidation, but tomato fruit effects are superior to those of pure carotenoid(s) (Canene-Adams *et al.*, 2007; Alshatwi *et al.*, 2010). In the light of this evidence, it is possible that other bioactive compounds present in tomato fruit contribute to the anti-cancer and anti-inflammatory properties of tomato fruit. Peptides and proteins present in tomato fruit have received little attention even though such compounds might have biological activity in human cells.

## CYSTINE-KNOT MINIPROTEINS

Cystine-knot miniproteins are members of a large family of small proteins widespread in plant and animal species; they display a multitude of therapeutically useful biological activity. Cystine-knot miniproteins typically consist of less than 50 amino acids and they have a unique three-dimensional structure characterized by three intra-molecular

disulfide bonds forming a cystine-knot and a small tripled stranded β-sheet (Craik, 2001). The cystine-knot acts as structural scaffold for the whole protein or for one of its domains conferring to the cystine-knot miniproteins a compact and remarkably stable structure in the face of extreme pH, chemical and thermal denaturation, and proteolytic attack (Ireland *et al.*, 2006; Werle *et al.*, 2007). Naturally occurring cystine-knot miniproteins, despite their similar molecular structure, feature a multitude of different biological functions. In animals, cystine-knot miniproteins include factors that function as extracellular ligand and regulate numerous cellular functions such as cell growth and development (Vitt *et al.*, 2001). In plants, cystine-knot miniproteins are often involved in resistance to pathogens with the function of protease inhibitors or as elicitors of defence responses against pathogens (Norton and Pallaghy, 1998; Quilis *et al.*, 2007).

There are two classes of cystine-knots: growth factors cystine knot and inhibitor cystine-knots, which includes the cyclotide (cyclic cystine knot; Figure 1). The penetrating disulfide bond for the cystine-knot growth factors is Cys I-IV whereas it is Cys III-VI in the cystine-knot inhibitors and cyclic cystine-knot miniproteins.

The cysteine interlocked connectivity is necessary but not sufficient to ensure the membership of the family; indeed for example, each of the 4 domains of wheat germ agglutinin have such a connectivity but with a planar spiral topology (Wright, 1981) and so it is not a cystine-knot miniprotein.

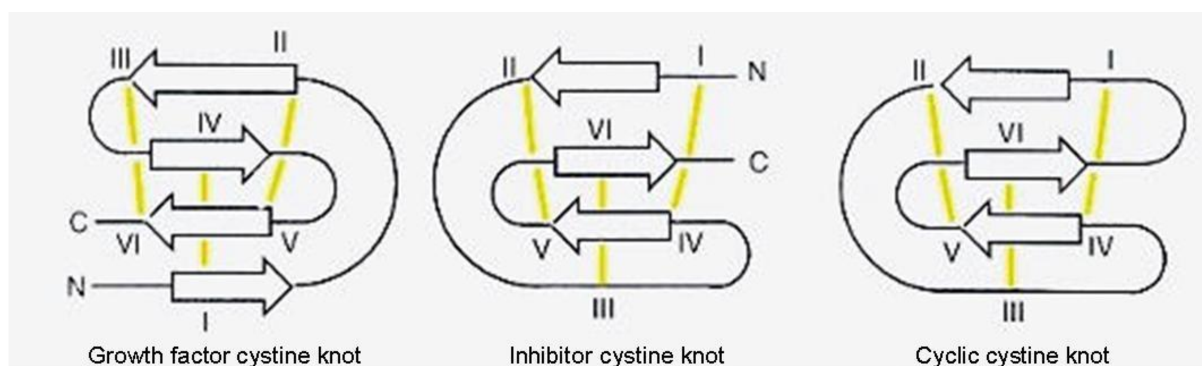


**Figure 1.** Schematic diagrams of the three groups of cystine-knot miniproteins. The β-strands are drawn as arrows, the cysteine residues are labeled I-VI from the N- to C-terminus and the disulfide bonds are colored in yellow. (from web site www.cyclotide.com/knots.html).

Cystine-knot miniproteins display a broad spectrum of therapeutically useful natural biological activities and several members of this family are marketed as therapeutics or are in clinical development (Gustafson *et al.*, 2004; Krause *et al.*, 2007). To extend the broad range of natural biological activities of cystine knot miniproteins to those with therapeutic relevance, several pieces of protein engineering research have been reported. The cystine-knot framework, owing to its inherent thermal and biological stability, is an attractive scaffold for the design of stable, functional small proteins for pharmaceutical applications. One of the major advantages of this scaffold lies in the fact that the cystine-knot core is a reproducible structural unit that can be functionalized by decoration with bioactive loop residues. Miniprotein loops tolerate the substitution of individual amino acids as well as the insertion of additional amino acids (Cwirla *et al.*, 1997; Krause *et al.*, 2007; Christmann *et al.*, 1999). Because the knottin scaffold retains its rigid structure upon loop modification, structure-based modelling and optimisation of miniprotein binding to the target protein is feasible.

Alternatively, the natural bioactivity of a given cystine-knot miniprotein can be used as a starting point for improvement of the pharmacologic properties. The main advantage of using cystine-knot miniprotein for pharmaceutical applications lies in the fact that high affinities (up to the picomolar range) and selectivities can be obtained. Moreover, many, but not all, cystine-knot miniproteins are stable against proteolytic attack and display high plasma stabilities in comparison with other proteins and peptides. They can be boiled, incubated at 65°C for weeks, or even placed in 1N HCl or 1N NaOH, without loss of structural and functional integrity (Werle *et al.*, 2007; Heitz *et al.*, 2008; Kimura *et al.*, 2009; Wang *et al.*, 2009). Many cystine-knot miniproteins are *per se* resistant against serum proteases or even against intestinal proteases and therefore could be delivered by mouth (Werle *et al.*, 2007; Werle *et al.*, 2008; Werle *et al.*, 2006).

## GROWTH FACTORS CYSTINE-KNOT

The family of growth factors cystine-knot consists of four distinct sub-families, nerve growth factor (NGF; McDonald *et al.*, 1991), transforming growth factor-beta (TGF-β; Schlunegger and Grütter, 1992; Daopin *et al.*, 1992), platelet-derived growth factor (PDGF; Oefner *et al.*, 1992) and the glycoprotein hormones (GPHs). These homo- and

hetero-dimeric proteins have a diverse range of effects on cell survival, proliferation and differentiation which are mediated via interactions with quite distinct receptor families (Figure 2).

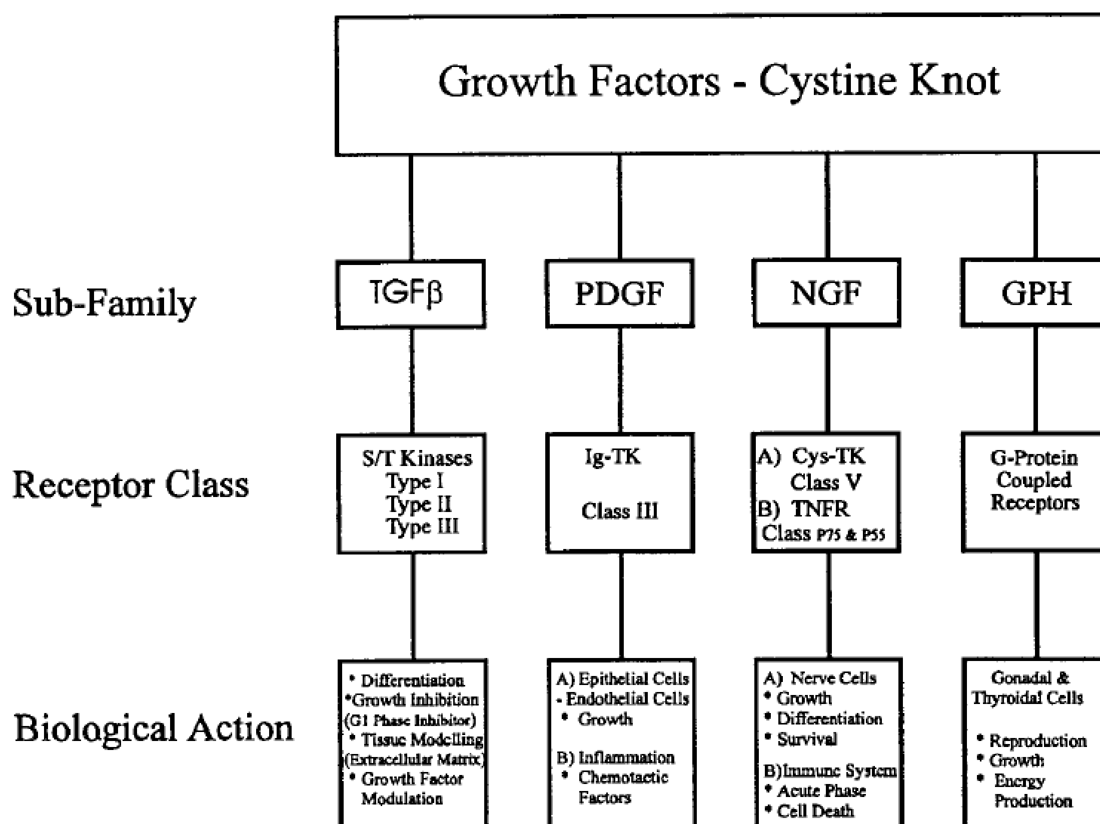


**Figure 2.** Classification of the growth factor cystine-knot superfamily. The abbreviations used are: TGFβ, transforming growth factor-beta;PDGF, platelet-derived growth factor; NGF, nerve growth factor; GPH, glycoprotein hormones; S/T, threonine/serine; TK, tyrosine kinase; Ig, immunoglobulin-like; Cys, cysteine-rich; TNFR, tumour necrosis factor receptor. (from Hearn and Gomme, 2000)

Comparison of the primary structures of the different members of these four subsets indicates that, with the exception of residues associated with the cystine-knot motif, there is very little sequence similarity. The N- and C-terminal and loop regions of the family members vary both in size and conformation.

The growth factors have acquired differences in the loops conformation during the course of evolution that presumably serve to modulate their biological specificity or that enable them to trigger cooperative conformational changes in their cognate receptors as part of the process of signal transduction (Pallaghy *et al.*, 1994).

# INHIBITOR CYSTINE-KNOTS OR KNOTTINS

The knottins are widespread miniproteins present in many species and featuring various biological actions such as protease inhibitory, anti-microbial, insecticidal, cytotoxic, anti-HIV or hormone-like activity (Chiche *et al.*, 2004). They share the unique knotted topology of three disulfide bridges forming a knot. This scaffold was first discovered in 1982 in PCI, a carboxypeptidase inhibitor from potato (Rees and Lipscomb, 1982). It has since been observed in a great number of unrelated proteins families including, e.g. toxins from plants, bugs, molluscs or arachnids, or anti-microbials from plants, insects or arthropods. Proteins sharing this scaffold were referred to as knottins (Le Nguyen *et al.*, 1990) or inhibitor cystine-knots (Pallaghy *et al.*, 1994) or even simply as cystine-knots. The most populated knottin families are conotoxins (446 sequences), spider toxins (593 sequences) and cyclotides (165 sequences). A complete list of the knottin families is listed in Table 3.

**Table 3.** List of the knottin types (from http://knottin.cbs.cnrs.fr/Knottins.php).

| ***Type*** | ***N° of sequences*** | ***Type*** | ***N° of sequences*** |
|---|---|---|---|
| Agouti-related | 118 | Maize | 8 |
| Alpha-amylase inhibitor | 1 | Metallo carboxypeptidase inhibitor | 13 |
| Algae | 1 | Plant antimicrobial | 13 |
| Bacteria | 3 | Plant defensin | 2 |
| Bug | 3 | Plant toxin | 28 |
| Conotoxin1 | 440 | Scorpion1 | 7 |
| Conotoxin2 | 5 | Scorpion2 | 47 |
| Conotoxin3 | 1 | Serine protease inhibitor1 | 36 |
| Cyclotide | 165 | Serine protease inhibitor2 | 4 |
| Fungi1 | 12 | Spider | 593 |
| Fungi2 | 20 | Sponge | 1 |
| Gumarin-like | 1 | Trematoda | 12 |
| Horsehoe crab | 5 | Trichoplax | 1 |
| Insect1 | 11 | Virus1 | 24 |
| Insect2 | 22 | Virus2 | 24 |

Polypeptides containing the inhibitor cystine-knot motif are of low molecular weight and may lack the conformational versatility necessary to participate in cooperative

conformational changes upon interaction with their target proteins, behaving therefore mainly as inhibitors (Pallaghy *et al.*, 1994).

The inhibitor cystine-knot motif is composed of an anti-parallel, triple stranded β-sheet stabilized by a cystine-knot, as shown schematically in Figure 3.

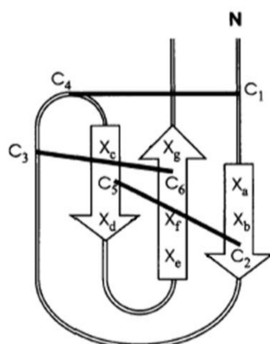


**Figure 3.** Schematic diagram of the inhibitor cystine knot structure. C represent a half-cystine residue and X represents any other residue. (adapted from Pallaghy *et al.*, 1994).

The only highly conserved signature of members of the knottin family is the space between adjacent cysteine residues and the mode of disulfide bond connectivity. The backbone loops, however, are variable according to length and amino acids sequence.

A large family of cystine-knot miniproteins (cyclotides), exclusively found in plants, has the unusual feature of a head-to-tail cyclized peptide (Craik *et al.*, 2007; Craik *et al.*, 2006).

Both open chain and circular miniproteins containing the cystine-knot motif are remarkably stable in the face of extreme pH, chemical and thermal denaturation and proteolytic attack (Ireland *et al.*, 2006; Werle *et al.*, 2007). This is probably a result of the enormous conformational rigidity that is introduced by covalent disulfide linkage of the knotted core (Colgrave and Craik, 2004; Krätzner *et al.*,2005).

## CYCLOTIDES

The cyclotides (Craik *et al.*, 1999) are a remarkable family of small plant-derived proteins that have the unique feature of a circular protein backbone. They typically contain 28-37 amino acids linked in continuous circle of peptide bonds and thus contain no free N- or C-terminal. The cyclotides are extremely resistant to proteolysis and are remarkably stable. These features have led to suggestions that this protein family makes an excellent template for drug design application (Craik *et al.*, 2002). Cyclotides with haemolytic (Schöpke *et al.*, 1994), cytotoxic (Lindholm *et al.*, 2002), anti-HIV (Gustafson *et al.*, 2004) and anti-fouling (Göransson *et al.*, 2004) activities have also been described.

They also have exciting potential applications in agriculture because they are potent insecticidal agents (Jennings *et al.*, 2001).

## POTATO CARBOXYPEPTIDASE INHIBITORS

A cystine-knot protease inhibitor was first discovered in 1980 in potato and it was shown that this miniprotein acts as a carboxypeptidase inhibitor (Rees and Lipscomb, 1980). The potato metallo carboxypeptidase inhibitor (PCI) has been by far the best characterized at the protein level (Avilés *et al*., 1993). PCI is a small, 39 amino acid-long, globular protein that competitively inhibits several metallo carboxypeptidases with a $K_i$ in the nanomolar range (Hass *et al*., 1979a). The NMR three-dimensional structure of PCI in aqueous solution (Clore *et al.*, 1987) and the crystal structure of its complex with carboxypeptidase A (Rees and Lipscomb, 1982) has been determined. The 27-residue globular core is stabilized by the three disulfide bridges and lacks regular secondary structures except for a short 5-residue helix and a very small β-sheet. From its hydrophobic central core protrudes a C-terminal tail (residues 35-39), which is inserted into the active site of the carboxypeptidase and shapes the primary binding site with the enzyme. A short stretch of core residues located around Trp28 constitutes the secondary binding site (Rees and Lipscomb, 1982). Several studies have demonstrated the therapeutic properties of PCI in different diseases. Administration of PCI reduced tissue factor-induced renal microthrombosis in rats (Muto *et al.*, 2003); moreover, *in vivo* anti-thrombotic efficacy of PCI was demonstrated in murine model: administration of PCI inhibits thrombin activatable fibrinolysis inhibitor (TAFI; also known as plasma procarboxypeptidase B), the molecule that renders a fibrin-containing thrombus less sensitive to lysis (Wang *et al.*, 2006).

PCI could be a therapeutic agent not only for its inhibitory activity, but also when exploited for its knotted structure. In particular, it has been shown that PCI display structural homology with mammalian epidermal growth factor (EGF), which makes this molecule an EGF antagonist with potential significance as an anti-tumor agent: PCI inhibits pancreatic adenocarcinoma cell growth by binding to the epidermal growth factor receptor (EGFR) (Blanco-Aparicio *et al.*, 1998; Sitjà-Arnau *et al.*, 2005).

Recently it has been demonstrated that potato contains three PCI isoinhibitor families (I, II and III) that are closely related. The predominant form (II) is a mixture of two polypeptides (IIa and IIb) with the difference in that IIa possessed an additional residue of glutamine (Hass *et al*., 1975). Isoinhibitor I was shown to be identical to II except for two replacements (S30A and R32G). These replacements had no significant effect on apparent $K_i$ values towards either carboxypeptidase A or B. Isoinhibitor III, which was identical to II except that it lacked the amino terminal pyrrolidone carboxylic acid and the following glutamine residue, was also functionally indistinguishable from II (Hass *et al*., 1979a). The relative amount of each of three isoinhibitor families was estimated in extract of leaves, stems, sprout and tuber and it was demonstrated that very few differences in distribution could be found in the potato plant, although tuber contained the highest level. Furthermore, the three isoinhibitor families account for essentially all of the carboxypeptidase inhibitor activity in the potato tuber. However, PCI activity is found throughout the plant and thus the function of this peptide is presumably not tissue-specific (Hass *et al*., 1979b).

## TOMATO CARBOXYPEPTIDASE INHIBITORS

In 1981 Hass and Hermodson published the sequence of a tomato carboxypeptidase inhibitor, but it was only in 1991 that Martineau *et al.* identified and characterized the cDNA clone of the tomato metallo carboxypeptidase inhibitor (TCMP-1) screening a *Solanum lycopersicum* cv UC82 ovary cDNA library. The clone contained an open reading frame coding for the 37 amino acids mature TCMP protein sequence identified, as well as an additional N-terminal sequence of 32 amino acids and an extension of 8 amino acids beyond the C-terminal of the published TCMP amino acid sequence (Hass and Hermodson, 1981). The N-terminal 32 amino acid region contains a typical hydrophobic signal sequence associated with transport of the nascent polypeptide across the rough endoplasmic reticulum (von Heijne, 1983). The polypeptide extension beyond the carboxy-terminus of the mature protein is also quite hydrophobic and it is similar to sequences found in a vacuolar protein of wheat germ (Raikhel and Wilkins, 1987) and in a barley lecithin (Lerner and Raikhel, 1989). For the C-terminal of barley lecithin it has been demonstrated its importance for the vacuolar sorting of the peptide (Wilkins *et al.*, 1990; Bednarek *et al.*, 1990). Furthermore, PCI is accumulated in the vacuoles of potato

leaf cells (Holländer-Czytko *et al.*, 1985); so the C-terminal of TCMP could be necessary for the vacuolar sorting. Alternatively, the C-terminal could inactivate the TCMP until the plant has reached a precise development stage or until the TCPI has reached its sub-cellular localization.

The function of TCMP-1 in plant is still not well understood, but it is considered, like other carboxypeptidase inhibitors, to be a potential protective agent against plant predators and pest by significantly lowering the digestibility of the plant organs where it is present (Bayés *et al*., 2006)

Active TCMP-1 is present in tomato leaves in response to wounding and elicitors and it is synthesized with the same temporal induction as other wound inducible proteinase inhibitors that defend the plants against herbivores' attacks (Díez-Díaz *et al.*, 2004). TCMP-1 accumulation is highest in tomato ovary tissue during the period immediately after the opening of flowers (anthesis) (2%-5% of the total cellular protein) and is present at a very low level in ripe fruit (<0.4% of the total cellular protein) (Martineau *et al.*, 1991)

In 1989 the differential screening of a cDNA bank constructed from ripe tomato fruit mRNA (*Solanum lycopersicum* UC82) allowed the isolation of the cDNA clone 2A11. The open reading frame of 2A11 encoded a sulfur-rich polypeptide 96 amino acids in length (TCMP-2) (Pear *et al.*, 1989). The putative protein sequence showed a very hydrophobic region at the N-terminal end which may indicate that the gene product is transported into a compartment other than the cytoplasm (Perlman and Halvorson, 1983).

The gene encoding for TCMP-2 is fruit specific and shows no detectable expression in mRNA from roots, stems or leaves. The TCMP-2 message is approximately 1% of the poly($A^+$) mRNA in ripe tomato fruits (Pear *et al.*, 1989).

## ANGIOGENESIS

Angiogenesis, or neovascularisation, is the process of generating new capillary blood vessels. Blood vessel formation occurs both in physiologic and pathologic conditions,

and it supports tissue growth and organ function in development, physiology and disease. An insufficient supply of nutrient and oxygen prompts the formation of new vessels from the walls of existing vessels in a process termed angiogenic sprouting. The principal cells involved are endothelial cells, which line all blood vessels and constitute virtually the entirety of capillaries. Hypoxic tissues secrete growth factors and chemokines that stimulate the endothelial cells to escape from their stable location by breaking through the basement membrane. Once this is achieved, endothelial cells migrate towards the angiogenic stimuli and jointly coordinate sprouting, branching and new lumenized network formation, until supply meets demand and quiescence can be re-established (Fraisl *et al.*, 2009).

Once it is initiated by environmental growth factor signals, the sprouting process is spearheaded by leading endothelial tip cells (Gerhardt *et al.*, 2003). In response to vascular endothelial growth factor-A (VEGF-A), these specialized endothelial cells situated at the tips of vascular sprout extend long filopodia that respond to extracellular VEGF-A gradients through tyrosine kinase and other receptors and lead to directed migration of tip cells. The endothelial cells that follow the tip cells, termed stalk cells, produce fewer filopodia and instead proliferate when stimulated with VEGF-A (Gerhardt *et al.*, 2003). They also form the vascular lumen (Iruela-Arispe and Davis, 2009), and they establish adherens and tight junctions to maintain the integrity of the new sprout (Dejana *et al.*, 2009) and to establish luminal/abluminal polarity, which, in turn, leads to basal lamina deposition and mural cell recruitment/attachment. It has been demonstrated that endothelial tip and stalk cells differ in their gene expression profile and that these differences are quantitative and most prominent at the mRNA level. (Claxton and Fruttiger, 2004; Siekmann and Lawson, 2007; Tammela *et al*., 2008). These quantitative differences in gene expression support the idea that tip and stalk cells have specialized functions during sprouting angiogenesis. Each new sprout eventually connects with adjacent sprouts via the tip cell establishing a simple vascular network. The vascular lumen is then established via formation of vacuoles, which eventually fuse with those of adjacent endothelial cells to generate a seamless tube with an intracellular lumen and thus establish flow in the new vascular loop (Blum *et al.*, 2008; Leslie *et al.*, 2007). Establishment of flow and recruitment of basement membrane and mural cell contribute to the remodelling and maturation of the new vascular connection (Jain,

2003; Jones *et al*., 2006; Lucitti *et al.*, 2007). Flow-dependent tissue oxygenation finally downregulates paracrine VEGF-A production, and thus helps establish a quiescent state for the new vessels.

Not all connections remain stable, however, as extensive remodelling reshapes the primitive plexus (Fruttiger, 2007). Vascular network reorganization demonstrates the capacity to alter its branching pattern by means of alternative forms of nonsprouting angiogenesis (Kurz, 2000), for example by intussusceptions, i.e. the formation of transvascular tissue pillars (Burri and Tarek, 1990), or by the phenomenon of 'pruning', i.e. obliteration and removal of branches from a bifurcation (Baffert *et al.*, 2006; Hughes and Chang-Ling, 2000). All these events are regulated by hemodynamic forces and crosstalk between the endothelial cells, mural cells, and the tissue environment (Djonov *et al.*, 2002; Jain, 2003; Lucitti *et al.*, 2007).

What controls the specification of endothelial tip and stalk cells? How is tip cell migration and protrusive activity regulated? What controls the number of stalk cells and their proliferation? How do tip cells communicate during the formation of new connections? And what determines which connections will regress during pruning? All of these processes are tightly controlled by pro-angiogenic and anti-angiogenic factors, and are driven by a cocktail of growth factors and pro-angiogenic cytokine (Klagsbrun and D'Amore, 1991; Lee *et al*., 2005). The list of factors capable of eliciting an angiogenic response is steadily growing; some of these factors take a centre stage position, whereas others play more auxiliary roles (Risau, 1997). VEGF and its receptors play a critical roles in the processes of angiogenesis promoting endothelial cell differentiation, migration, and proliferation; controlling endothelial cell-cell junctions; suppressing apoptosis, and more. Many other pathways, including the epidermal growth factor receptor (EGFR), regulate the angiogenic response. Over the past few years, it has become clear that the EGFR signaling pathway plays a key role in coordinating multiple aspects of endothelial behaviour during vessel patterning and thus in shaping the formation and remodelling of the vascular network. For instance, VEGF can induce other pro-angiogenic factors, such as heparin-binding epidermal growth factor (HB-EGF) which promotes angiogenesis in endothelial cells via EGFR activation (Mehta and Besner, 2007). Furthermore, the angiotensin II-mediated cross-activation of EGFR is

responsible for neo-angiogenesis in endothelial cells and for the expression of VEGF (Fujiyama *et al.*, 2001).

## ANGIOGENESIS IN CANCER

As has been said before, angiogenesis is the formation of new blood vessels from pre-existing vessels to form capillary networks. It is a fundamental event of physiological processes, like organogenesis, wound healing and muscle growth (Carmeliet, 2005) and involves a complex balance of positive and negative regulators, including VEGF which is one of the most important pro-angiogenic factor.

Unfortunately, angiogenesis takes also place in pathological situations and it is a prerequisite for sustained growth of primary tumors and metastases. The angiogenic process in the tumor microenvironment involves the complex interplay of free angiogenic growth factors with their cognate receptors and endothelial cell activation, proliferation, and migration leading to a vascular remodelling. The VEGF family has been the most extensively studied among pro-angiogenic factors. (Epstein, 2007; Shibuya and Claesson-Welsh, 2006). The VEGF family includes 6 glycoproteins (VEGF-A to E and placental growth factor) and 3 tyrosine kinase receptors (VEGFR1 to 3). VEGF-A promotes angiogenesis through enhancement of permeability, activation, survival, migration, invasion, and proliferation of endothelial cells (Hicklin and Ellis, 2005). VEGFR1 and VEGFR2 mediate the effects of VEGF-A (Ferrara *et al.*, 2003). Recent studies suggest that VEGF-A is the main ligand involved in tumor angiogenesis (Kim *et al.*, 1993; Ferrara and Gerber, 2001; Ferrara, 2002). A direct effect has been shown of VEGF-A on tumor cell proliferation via a mechanism thought to involve the Akt/mTOR pathway (Trinh *et al.*, 2009); VEGF-A regulates the invasiveness of cancer cells by altering the expression of matrix metalloproteinase-2 (Zhang *et al.*, 2006). Moreover, the tumor cell and its supporting infiltrating macrophages and mesenchymal cells have been demonstrated to secrete VEGF-A (Liang *et al.*, 2006), which contributes to increased tumor growth and metastasis.

Angiogenesis and the molecular determinants of the angiogenic cascade have been characterized over the years, and it has emerged that their role raised great importance not only for tumors, but also for a number of non-oncological diseases, such as

rheumatoid arthritis (Szekanecz *et al*., 2009), psoriasis (Chua and Arbiser 2009), proliferative retinopathies (Sapieha *et al*., 2010), diabetic retinopathies (Crawford *et al*., 2009) and age-related macular degeneration (Novak, 2006).

## ANGIOGENESIS AS THERAPEUTIC TARGET

Being one of the hallmarks of cancer, anti-angiogenic therapy in cancer has become very popular. This idea was introduced 40 years ago by Folkman (Folkman, 1971), but even if specific angiogenic inhibitors have been discovered, challenges still exist in the clinical application of these inhibitors. The most impressive anti-cancer results today are with agents targeting VEGF/VEGF receptor (VEGFR) pathway.

The most widely investigated anti-VEGF ligand agent is *bevacizumab* (BEV). BEV is a recombinant humanized monoclonal antibody that binds and neutralizes all biologically active isoforms of VEGF (Ferrara *et al.*, 2004). BEV is now in clinical use and effective toward some types of cancer (Folkman, 2006). Other monoclonal antibodies directed against VEGF are in clinical trials.

Besides VEGF-ligand-binding anti-angiogenic agents, there are VEGFR tyrosine kinase inhibitors. For example *cediranib*, a highly selective and potent oral tyrosine kinase inhibitor of VEGFR1, VEGFR2, VEGFR3 (Wedge *et al*., 2005) that is in phase I evaluation (van Cruijsen *et al*., 2010) or *semaxinib*, a tyrosine kinase inhibitor with activity against VEGFR2 that is able to reduce microvessel density and tumor growth in preclinical tumor model with high VEGF expression (Holtz *et al.*, 2008).

Despite these promising data, some combination trials have resulted in very disappointing results. The inhibitors have been seen to cause serious side effects (Elice *et al.*, 2009; Daher and Yeh, 2008), and in a recent preclinical study of a therapy with *metronomic paclitaxel* in combination with the VEGFR2 inhibitors *semaxinib*, researchers found that those agents showed an additive effect in tumors with low VEGF expression, while they observed an antagonism in tumors with high VEGF expression (Holtz *et al.*, 2008). These experiments suggest that a better knowledge of various molecular pathways implicated will help us to investigate the optimal combination partners.

In conclusion, anti-angiogenic treatment for cancer is looking very promising but it still requires the identification of new angiogenesis inhibitors with limited toxicity and a broader efficiency.

## EGF AND EGFR

Human epidermal growth factor (EGF) is a 53 amino acids single-chain polypeptide (6 kDa) that arises from the proteolytic cleavage of a large (1207 amino acids) integral membrane protein precursor (Carpenter and Cohen, 1990). This growth factor stimulates the proliferation of epithelial cells, inhibits gastric acid secretion, and is involved in wound healing. EGF is closely related structurally to transforming growth factor-alpha (TGF-α), which bind to epidermal growth factor receptor (EGFR).

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor of the ErbB family, which is composed of four closely related transmembrane tyrosine kinases: ErbB1 (EGFR), ErbB2 (HER2/*neu*), ErbB3 and ErbB4. These receptors can form both homo- and heterodimers in a manner that is dependent on the combination of receptors present, the stimulatory ligand, and the intracellular signaling pathway activated (Riese, 1995). This allows them to affect, positively and negatively, a wide variety of cellular functions.

The EGFR is composed of three regions: the extracellular ligand domain, a transmembrane region, and the intracellular protein tyrosine kinase domain (Ennis *et al.*, 1991). There are a number of EGFR ligands, including EGF, TGF-α, amphiregulin, heparin-binding EGF, betacellulin, and epiregulin (Salomon *et al.*, 1995). Epidermal growth factor and TGF-α are believed the most active EGFR ligands. These ligands function not only in a paracrine manner, but can also be secreted from tumor cells in an autocrine manner, thus enhancing their effects (Ruck and Paulie, 1998). Binding of ligands to the EGFR induces receptor dimerization, which leads to a cascade of cellular events. Upon EGFR dimerization, multiple residues of cytoplasmic kinase domain are autophosphorylated and several downstream adaptor protein are recruited under the plasma membrane, including GRB2, Shc or Dok-R. These adaptors mediate the activation

of different signal transduction pathways depending upon the type of ligand, levels of receptor expression and partner of EGFR dimerization. One of the most studied downstream pathways include the Ras/ERK signalling cascade (Figure 4 ).

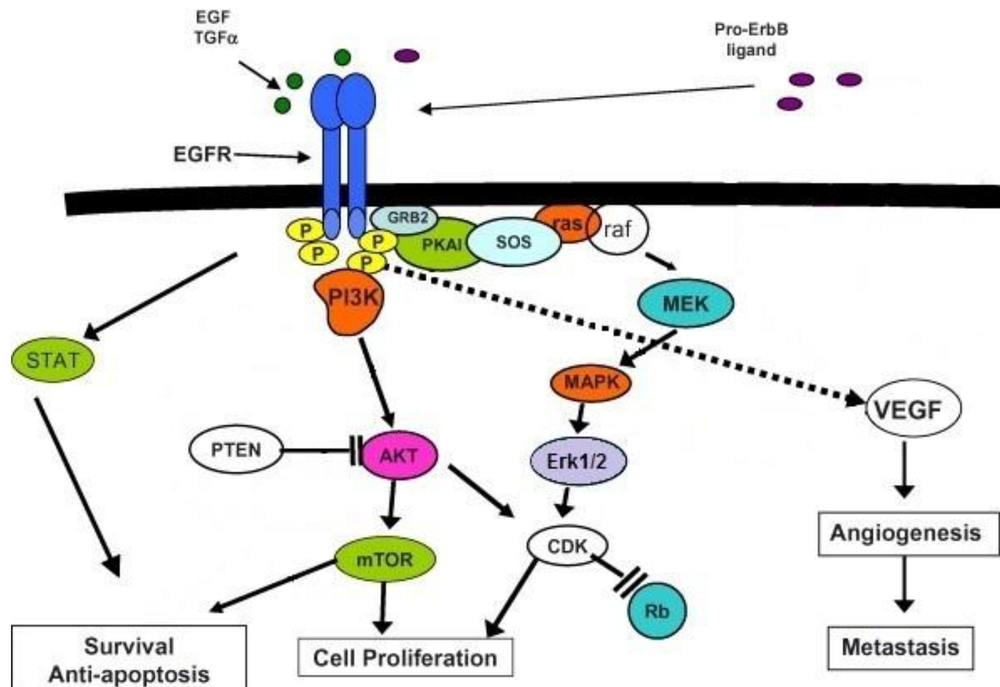


**Figure 4.** Schematic representation of EGFR downstream signaling pathway.(adapted from Bianco *et al.*, 2007)

The key player in EGFR-dependent Ras activation is the adaptor protein Grb2, which associates to the receptor, either directly or indirectly, by binding to EGFR linked and tyrosine phosphorylated Shc (Batzer *et al.*, 1994; Lowenstein *et al.*, 1992). It has been suggested that association of Shc with EGFR, which leads to its tyrosine phosphorylation and to the recruitment of Grb2, is the critical step in EGF-dependent induction of the Ras/MAPK pathway (Waters *et al*., 1996). Pre-associated with Grb2 is Sos, a guanine nucleotide exchange factor. Translocation of Grb2/Sos complex to the receptor in the plasma membrane facilitates the interaction of membrane-associated Ras with Sos, resulting in the activation of Ras small G protein. Activated Ras leads to the activation of the Raf-kinase that phosphorylates and activates the mitogen-activated protein kinases (MAPKs) (Voice *et al*., 1999; Marais and Marshall, 1996). MAPKs is a superfamily of protein serine-threonine kinases, which includes the extracellular signal regulated kinases (ERKs), the c-Jun terminal kinase (JNKs) also know as stress-activated protein

kinases (SAPKs) and p38-mitogen-activated protein kinases (Johnson *et al*., 2005). MAP kinases can translocate into the nucleus and phosphorylated transcription factors making them active inducing gene transcription, leading to increased levels of inhibitors of apoptosis proteins (IAPs) and anti-apoptotic Bcl-2 family members (Lin *et al*.,2002). Finally Erk1/2 kinases positively regulate cell proliferation by activating the major transcription factors associated with cell proliferation, such as c-Myc, and ribosomal subunit kinase (RSK) family isoforms (Murphy *et al*., 2002; Gavin and Nebreda, 1999).

The transduction pathway primarily involved in the regulation of cell proliferation and apoptosis is represented by the PI3K/Akt pathway. The EGFR C-terminal intracellular domain provides a docking site for the p85 subunit of PI3K either directly or indirectly through binding to protein adaptors such as Grb2 (Stover *et al*., 1995). Upon activation, PI3K generates phosphatidyl-inositol-3,4,5-tris-phosphate (PIP3) which recruits and activates the serine-threonine kinase Akt. Phosphorylated Akt controls cell survival through phosphorylation of several downstream targets, such as apoptotic proteins of the Bcl-2 family (Song *et al*., 2005), transcription factors which, in turn, cause an increased transcription of anti-apoptotic genes (Song *et al*., 2005; Kane *et al.*, 1999), and protein kinases or causing their inactivation, as in the case of Gsk-3β involved in the regulation of cell metabolism, or causing their activation, as for mTOR implicated in cell survival (Chan, 2004; Sun *et al*., 2005). Again, the final result is a decreased pro-apoptotic gene expression and consequent increased cell survival.

In addition to the Akt survival pathway, EGFR is able to regulate STATs pathway (Andl *et al.*, 2004; Kloth *et al.*, 2003). In fact, EGFR activation induces phosphorylation of STAT1, which in turn initiates formation of complexes, causing the translocation of STAT proteins to the nucleus and consequent regulation of gene expression and cell survival (Andl *et al.*, 2004; Leaman *et al.*, 1996).

Additional biological responses of EGFR activation can result in proliferation of cancer cells with enhanced metastatic potential.

## RELATIONSHIP AMONG EGFR, CANCER AND ANGIOGENESIS

Tumor development and progression depend mainly on cellular changes like over-expression of oncogenic tyrosine kinase receptors (Hanahan and Weinberg, 2000). The activity of the EGFR, that is a tyrosine kinase receptor of the ErbB family, is abnormally elevated in most human solid tumors and has been associated with progression and poor prognosis (Salomon *et al*., 1995; Brabender *et al.*, 2001). Various mechanisms are likely to be involved in aberrant EGFR signaling: production of EGFR-ligands by tumor cells or stromal cells in their environment, high expression of EGFR associated with enhanced sensitivity for EGF-like ligands, and constitutively active EGFR mutations that lead to ligand-independent activation of the receptor (Hirata *et al.*, 2002). Up-regulated EGFR signaling is known to initiate a cascade of events leading to cell proliferation, migration, invasion (Mendelsohn and Dinney, 2001) and blocking of apoptosis (Kulik *et al.*, 1997). So, EGFR is expected to be a valuable molecular target for effective anti-cancer agents. *In vitro* experiments with therapeutic agents against EGFR induce anti-proliferative effects in tumor cells cultured. This most frequently results in cytostatic effects, more than cytoreduction and apoptosis. In contrast, in several *in vivo* models anti-EGFR therapy frequently induces tumor regression, which may not be solely explained by inhibition of the molecular target. (Hanahan and Folkman, 1996). This discrepancy suggests that EGFR signaling affects both biological characteristics of the tumor cell and tumor-host interactions like angiogenesis. Angiogenesis is essential for tumor growth and metastasis formation and the VEGF is a key stimulator of this process. Petit *et al*. reported in 1997 that the oncogeneity of the EGFR might partially be mediated through promotion of angiogenesis by up-regulating VEGF (Petit *et al.*, 1997). Since then there has been growing evidence that stimulation or inhibition of EGFR also has significant consequences for tumor-induced angiogenesis (Yen *et al*., 2002; Ellis, 2004). Direct EGFR angiogenic effects have been demonstrated by Hirata and colleagues (Hirata *et al.*, 2002). It has been shown that human microvascular endothelial cells express EGFR (ErbB1). Stimulation of these cells with EGF or TGF-$\alpha$ induced tube formation. For a great variety of tumor cells it has been demonstrated that activation of the EGFR pathway increases the production of angiogenic molecules. EGF stimulation of glioma cells consistently increased the production of VEGF by these cells (Goldman *et al.*,

1993); EGFR and HER-2/*neu* have been identified as important regulators of gastric cancer growth and angiogenesis, which in part involves up-regulation of VEGF-A (Allgayer *et al.*, 2000). In a bladder carcinoma cell line, EGF increased the production of VEGF and bFGF (Perrotte *et al.*, 1999). In gastric and pancreatic cell lines, EGF stimulation also caused an enhanced production of neurophilin-1, a co-receptor of VEGFR2, increasing the affinity of specific isoforms of VEGF to VEGFR2 (Parikh *et al.*, 2003). Under EGF stimulation A431 cells (human epithelial carcinoma cell line), which express high levels of EGFR, produce VEGF and IL-8, both positive regulators of angiogenesis that stimulate endothelial cells growth through paracrine mechanisms (Hirata *et al.*, 2002). Furthermore, EGF induces VEGF in many cell lines through increased VEGF mRNA transcription (Maity *et al.*, 2000; Pore *et al.*, 2003). EGFR stimulation activates many downstream signaling pathways including the PI3K/Akt pathway (Dutta and Maity, 2007). Activated Akt increases expression of a key transcription factor, hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) (Zhong *et al*., 2000; Zundel *et al*., 2000). One of the many transcriptional targets of HIF-1$\alpha$ is the VEGF gene. Conversely, pharmacological inhibition of EGFR can decrease VEGF expression and consequently angiogenesis in many tumor type (Ciardiello *et al.*, 2001; Huang *et al.*, 2002; Perrotte *et al.*, 1999; Petit *et al.*, 1997; Pore *et al.*, 2006).

Based on this evidence, it is clear that there is a connection between EGFR signaling and VEGF expression, which means a tight correlation between EGFR and angiogenesis.

## EGFR AS THERAPEUTIC TARGET OF CANCER ANGIOGENESIS AND TUMOR GROWTH

In cancer anti-angiogenic therapy has become very popular, but only few VEGFR inhibitors are now in clinical use because most of them seem to cause serious side effects (Elice *et al.*, 2009; Daher and Yeh, 2008). Identification of new angiogenesis inhibitors with limited toxicity and a broader efficiency is needed and the idea is that agents inhibiting new vessel formation and blood supply can have anti-cancer effects.

EGFR and VEGFR share common downstream signaling pathway and several preclinical studies have provided evidence for either direct or indirect angiogenic effects of EGFR signaling. Multiple therapeutic strategies have been developed to target EGFR, including

monoclonal antibodies, tyrosine kinase inhibitors, ligand-toxin conjugates, antagonist of EGF/EGFR and antisense oligonucleotides. At this time there is no evidence showing superiority of one approach over another.

Anti-EGFR monoclonal antibody (MAbs) binds to the extracellular domain of the EGFR, which interferes with the binding of endogenous ligands, most importantly, EGF and TGF-$\alpha$. After binding, the receptor-antibody complex is internalized, preventing receptor dimerization and autophosphorylation. A variety of anti-EGFR MAbs have undergone significant preclinical testing and a number are in clinical testing in a variety of tumor types. For example *cetuximab* reduces the production of VEGF and IL-8 by human transitional, pancreatic, colon and epidermoid (A431) cancer cells *in vitro* (Petit *et al.*, 1997; Perrotte *et al.*, 1999; Bruns *et al.*, 2000; Ciardiello *et al.*, 2000), whereas antibodies directed against the extracellular domain of ErbB2 resulted in a dose-dependent down-regulation of VEGF production by an ErbB2 positive breast cancer cell line (Petit *et al.*, 1997).

Tyrosine kinase inhibitors interfere with EGFR functioning after ligand binding by directly inhibiting tyrosine phosphorylation (Noonberg and Benz, 2000). *Erlotinb* and *gefitinib*, are the tyrosine kinase inhibitors most advanced in clinical development. *Erlotinb* treatment of various human tumor cells in vitro and mice bearing xenografts *in vivo* led to decreased HIF-1 alpha and VEGF expression (Pore *et al.*, 2006; Cerniglia *et al.*, 2009). Hirata and colleagues has demonstrated that treatment of human microvascular endothelial cells with *gefitinib* inhibited EGF-induced migration and tube formation (Hirata *et al.*, 2002).

Epidermal growth factor receptor ligand can be attached or conjugated to toxins, such as ricin or *Pseudomonas* exotoxin, to form ligand conjugates (Noonberg and Benz, 2000). These receptor-targeted strategies bind exclusively to the EGFR and allow lethal toxin to enter the cell and result in cell death, but this approach is not as widely studied as the anti-EGFR MAbs.

EGF antagonists can act as anti-angiogenic factors and as anti tumor-growth: a synthetic murine EGF is capable of blocking the angiogenic stimulus of native murine EGF in the chick VIM model (Nelson *et al.*, 1991); treatments with chimeric fusion protein that

contain granzyme B fused with TGF-α resulted in selective and rapid tumor cell killing (Dälken *et al.*, 2006); the EGF antagonist action of PCI as inhibitor of cancer growth has been the first reported example of an antagonistic analogue of human EGF (Blanco-Aparicio *et al.*, 1998). Another promising molecule is Argos, a natural secreted antagonist of EGFR identified in *Drosophila* (Freeman *et al*., 1992). Argos functions by directly binding and sequestrating growth factor ligands that activate EGFR. It mimics EGFR by using a bipartite binding surface to entrap EGF (Klein *et al*., 2008).

EGFR or TGF-α antisense oligonucleotides that bind to DNA or RNA have been employed to block the protein synthesis within the cell (Moroni *et al.*, 1992). Antisense therapies are in the very early stages of testing, and their clinical utility for cancer treatment is still to be discovered.

## POTATO CARBOXYPEPTIDASE INHBITOR AS EGF ANTAGONIST

Much effort is currently being devoted to finding new molecules that target transduction pathway (Levitzki, 1996), including antagonists that bind to growth factor receptors without activating them (Zumkeller and Schofield 1995; Groenen *et al*., 1994; Baselga and Mendelsohn, 1994). The potato carboxypeptidase inhibitor (PCI) is the first reported antagonistic analogue of human EGF that is able to bind to EGFR without eliciting the activation of the receptor (Blanco-Aparicio *et al.*, 1998). Blanco-Aparicio *et al.* demonstrated that PCI, in micromolar concentration, not only binds to EGFR competing with EGF, but it also inhibits all the steps in ligand-induced activation of EGFR by EGF: receptor dimerization, induction of tyrosine kinase activity and tyrosine transphosphorylation. Moreover, they show that PCI was able to inhibit the development of human adenocarcinoma tumors transplantated into nude mice without inducing any observable toxic side effects and that PCI has a long lasting inhibitory effect on the *in vitro* growth of pancreatic adenocarcinoma cell lines, which is maintained even when it is removed from the culture medium. Blanco-Aparicio *et al.* surmise that the antiproliferative effect of PCI is probably due to the fact that it is an EGF antagonist and that the effect of PCI on tumor growth could be attributable to its special topology.

# AIM OF THE RESEARCH

In recent years, several natural products have received great attention for their potential use in cancer prevention thanks to their various benefits, noticeably little or no toxicity and side effects, low cost and human acceptance. Several studies highlighted tomato as an important health-promoting fruit, rich in carotenoid compounds as well as vitamins, minerals, dietary fiber and phenols. The anti-tumoral protective role of tomato has been principally ascribed to its high content of antioxidant molecules, especially lycopene. However, just what is the active principle(s) is not completely clear yet, but the research has almost completely focused on secondary metabolites, whereas other molecules, for instance peptides and proteins present in tomato fruit, have received little attention. Anti-angiogenic treatment is a very effective strategy in cancer therapy although the identification of new angiogenesis inhibitors with limited toxicity and a broader efficiency are needed. Among several targets, inhibition of EGFR seems to represent a novel and promising approach.

This research aims to investigate the biological action on human cells of two tomato miniproteins which are normal components of our diet. The motivation for this research stems from the discovery that two tomato cystine-knot miniproteins (TCMPs), one expressed in the flower (TCMP-1) and the other in the mature fruit (TCMP-2), are similar in sequence and structure to a potato carboxypeptidase inhibitor (PCI) that has been shown to have anti-cancer properties. PCI, acting as EGF antagonist, inhibits tumor cell growth (Blanco-Aparicio *et al.*, 1998). Since EGF is one of the signalling molecules participating in the formation of new vessels, we must ask ourselves whether, TCMP-1 and TCMP-2, that can bind to the surface of human cells (endothelial cells, carcinoma cells; Cavallini, 2005), could also exert anti-angiogenic activity with limited cell toxicity.

The first part of the project was dedicated to the production of the recombinant tomato miniproteins TCMP-1 and TCMP-2 and the purification of the TCMP-2 from tomato fruit. The second part concerned the evaluation of the biological action of TCMP proteins on human cells, focusing on angiogenesis and on the investigation of the signal transduction pathways related to EGFR. The last part regarded the creation of chimeric gene constructs for tomato plant transformation aimed at increasing the content of TCMPs in mature tomato fruits.

# MATERIALS AND METHODS

## PLANT MATERIAL

UC82 tomato plants were grown in a greenhouse under a 10/14 hours light/dark cycle at 24°C and 18°C, respectively. UC82 is a typical cultivar used by the processing industry. Commercially available ripe tomatoes were used for the purification of fruit TCMPs.

## NORTHERN BLOT ANALYSIS

Total RNA was isolated with Trizol (Invitrogen Ltd, Paisley, UK) and 20 μg of total RNA were separated on 1% agarose-formaldehyde denaturing gels. The gels were blotted overnight on Hybond $N^+$ membrane (Amersham Biosciences, GE Healthcare Europe GmbH, Munich, Germany) in 10X SSC. The DNA probes were labeled with ($\alpha$-$^{32}$P)-dCTP using 'Ready to go DNA labeling beads (-dCTP)' (Amersham Biosciences, GE Healthcare Europe GmbH, Munich, Germany). Unincorporated nucleotides were removed with Probe G-50 micro columns (Amersham Biosciences, GE Healthcare Europe GmbH, Munich, Germany). The membranes were hybridised overnight at 42°C in ULTRAhyb buffer (Applied Biosystem/Ambion, Austin, TX, USA). $10^6$ cpm $ml^{-1}$ of labelled probe were added to the hybridization buffer. The membranes were washed 2 times in 2X SSC/0.1% SDS for 5 minutes and 2 times in 0.1X SSC/0.1% SDS for 15 minutes at 42°C. Autoradiography was then performed using Kodak X-AR5 film. For TCMP-1, TCMP-2 and actin mRNAs analysis, the DNA probes were obtained by PCR using the following primers:

TCMP-1:
forward 5'-ATGGCACAAAAATTTACTATCCTTTTCACC-3';
reverse 5'-GATTACATATCACACCCTAATGACATAATT-3';
TCMP-2:
forward 5'-TGAAGCTACTTCCCACAAATATTTTG-3';
reverse 5'-TCCCTTTATTCATATTCTTCACACC-3';
Actin:
forward 5'-CCCGTTCAGCAGTGGTGGT-3';
reverse 5'-TACGAGGGTTATGCTTTGCC-3'.

## CLONING AND EXPRESSION OF RECOMBINANT TCMP-1 AND TCMP-2

The DNA sequences corresponding to the coding regions of the mature TCMP-1 and the putative 44 amino acids of mature TCMP-2 protein were amplified by PCR using cDNAs as template. The upstream and downstream primers were designed in order to introduce at the 5′-terminal a restriction site for *Nde*I and a His$_6$-tag sequence and at the 3′-terminal end two stop codons and a restriction site for *Bam*HI. The primers sequences are listed as follow:

TCMP-1 forward:
5′-GGGAATTC*CATATG***CATCATCATCATCATCAC**CAGCAATATGATCCAGTTTGTCACAAAC
CT-3′;

TCMP-1 reverse:
5′-CG*GGATCC*TTATTAAACATAGGGCCCACATGTCCCCGCGAA-3′;

TCMP-2 forward:
5′-TCCTGTAAC*CATATG***CATCATCATCATCATCAC**ACAAATATTTTGGGACTTTGTAACGAA
CCT-3′;

TCMP-2 reverse:
5′-TG*GGATCC*TTATTAAGGCAACAGGTTGCATGTACGGTATGT-3′

(sequences for restriction sites are reported in italics, the His$_6$-tag sequence is in bold and sequences for stop codons are underlined). PCR products were subcloned using pGEM®-T Easy Vector System, (Promega GmbH, Mannheim, Germany) and checked by sequencing. The PCR products were double digested with *Nde*I-*Bam*HI and the resulting fragments were ligated into pET12b vector (Novagen, Merck Chemicals Ltd, Nottingham, UK). The recombinant plasmids were named pET12-T1 (for TCMP-1) and pET12-T2 (for TCMP-2). Origami B and BL21 pLysS (Novagen, Merck Chemicals Ltd, Nottingham, UK) *Escherichia coli* (DE3) competent cells were transformed with pET12-T1 and pET12-T2 plasmids, respectively. For the TCMP-1, the expression of recombinant protein was induced by 0.7 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 24°C for 5 hours. For the TCMP-2, the expression of the recombinant protein was induced by 0.4 mM IPTG at 37°C for 5 hours.

## CLONING AND EXPRESSION OF RECOMBINANT TCMP-1 AND TCMP-2 AS FUSION PROTEINS

The DNA sequences corresponding to the coding regions of the mature TCMP-1 and the putative 44 amino acids of mature TCMP-2 protein were amplified by PCR using cDNAs as template. The upstream and downstream primers were designed in order to introduce at the 5′-terminal a restriction site for *Sma*I and nucleotides to reform the thrombin cleavage site and at the 3′-terminal end two stop codons and a restriction site for *Bam*HI. The primers sequences are listed as follow:

TCMP-1 forward:
5′-*CCCGGG***GCAGC**CAGCAATATGATCCAGTTTGTCACAAACCT-3′;

TCMP-1 reverse:
5′-CG*GGATCC*TTATTAAACATAGGGCCCACATGTCCCCGCGAA-3′;

TCMP-2 forward:
5′-*CCCGGG***GCAGC**ACAAATATTTTGGGACTTTGTAACGAACCT-3′;

TCMP-2 reverse:
5′-TG*GGATCC*TTATTAAGGCAACAGGTTGCATGTACGGTATGT-3′

(sequences for restriction sites are reported in italics, nucleotides to reform the thrombin cleavage site are in bold and sequences for stop codons are underlined). PCR products were subcloned using pGEM®-T Easy Vector System, (Promega GmbH, Mannheim, Germany) and checked by sequencing. The PCR products were double digested with *Sma*I-*Bam*HI and the resulting fragments were ligated into pET43.1a vector (Novagen, Merck Chemicals Ltd, Nottingham, UK). The pET43.1a vector is designed for cloning and high-level expression of peptide sequences fused with the 491 amino acids Nus•Tag™ protein. Vector encoded sequence can be completely removed when cloning into the *Sma*I site by cleaving the Nus•Tag fusion protein with thrombin. The recombinant plasmids were named pET43-T1 (for TCMP-1) and pET43-T2 (for TCMP-2). BL21, Origami B and BL21 pLysS (Novagen, Merck Chemicals Ltd, Nottingham, UK) *Escherichia coli* (DE3) competent cells were transformed with pET43-T1 and pET43-T2 plasmids. The expression of recombinant protein was induced by 0.4 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 5 hours.

## CLONING AND EXPRESSION OF RECOMBINANT TCMP-2 IN PERIPLASMIC SPACE

The DNA sequences corresponding to the coding regions of the putative 44 amino acids of mature TCMP-2 protein were amplified by PCR using cDNAs as template. The upstream and downstream primers were designed in order to introduce at the 5′-terminal a restriction site for *Sal*I, a $His_6$-tag sequence and a enterokinase cleavage site sequence and at the 3′-terminal end two stop codons and a restriction site for *Bam*HI. The primers sequences are listed as follow:

TCMP-2 forward:
5′-*GTCGAC*G**CATCATCATCATCATCAC**GACGACGACGACAAGACAAATATTTGGGACTT TGTAACGAACCT-3′;

TCMP-2 reverse:
5′-TG*GGATCC*TTATTAAGGCAACAGGTTGCATGTACGGTATGT-3′

(sequences for restriction sites are reported in italics, the $His_6$-tag sequence is in bold, the enterokinase cleavage site is double underlined and sequences for stop codons are underlined). PCR products were subcloned using pGEM®-T Easy Vector System and checked by sequencing. The PCR products were double digested with *Sal*I-*Bam*HI and the resulting fragments were ligated into pET12b vector. The recombinant plasmid was named pET12s-T2 (for TCMP-2). BL21, Origami B and BL21 pLysS *Escherichia coli* (DE3) competent cells were transformed with pET12s-T2 plasmid. The expression of recombinant protein was induced by 0.4 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 5 hours.

## RECOMBINANT TCMP-1 AND TCMP-2 PRODUCTION CHECK

To verify the production and localisation of TCMP-1 and TCMP-2 recombinant proteins, cell cultures were centrifuged at 10000 xg for 10 minutes at 4°C. The cell pellets were resuspended in lysis buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% glycerol). Cell suspensions were lysed by lysozyme (0.1 mg $ml^{-1}$), by repeated freeze and thawing (3 times) and by mild sonication. DNase (20 μg $ml^{-1}$) was also added to cell suspensions in order to decrease the viscosity of the samples. The soluble and insoluble fraction were

recovered by centrifugation at 16000 xg for 20 minutes at 4°C and analyzed by gel electrophoresis.

## PURIFICATION OF RECOMBINANT TCMP-1 AND TCMP-2

For the isolation of TCMP-1 and TCMP-2 recombinant proteins, cell cultures were centrifuged at 10000 xg for 10 minutes at 4°C. The cell pellets were resuspended in lysis buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% glycerol). Cell suspensions were lysed by lysozyme (0.1 mg $ml^{-1}$), by repeated freeze thawing (3 times) and by mild sonication. DNase (20 μg $ml^{-1}$) was also added to cell suspensions in order to decrease the viscosity of the samples. The insoluble fraction, containing aggregated target protein (inclusion bodies) were recovered by centrifugation at 16000 xg for 20 minutes at 4°C. The pellets were washed three times with wash buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 50 mM 2-mercaptoethanol, 5 mM EDTA, 2% Triton). Two protocols were then applied. First, inclusion bodies were resuspended in the solubilisation buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 6 M guanidine hydrochloride, 50 mM 2-mercaptoethanol) for 1 hour and centrifuged 3000 xg for 10 minutes at room temperature. Denaturized inclusion bodies were loaded on a $Ni^{2+}$-loaded HiTrap Chelating column (Amersham Biosciences, GE Healthcare Europe GmbH, Munich, Germany) and recombinant proteins (His-tagged) were affinity attached. Renaturation of the TCMPs occurred in column with a decreasing gradient (0.1 ml $min^{-1}$) of guanidine hydrochloride, from 6 M to 0 M, and 2-mercaptoethanol, from 50 mM to 5 mM. Renaturated proteins were then eluted and the fractions were loaded on a polyacrylamide gel and stained. Fractions containing the miniprotein of interest were pooled, dialyzed and concentrated with Amicon stirred cells with a membrane 1 kDa cut off (Ultrafiltration Membranes, Millipore).

In the second protocol applied, inclusion bodies were resuspended in the solubilisation buffer (20 mM Tris-HCl pH 8.5, 6 M guanidine hydrochloride, 30 mM DTT) for 5 hours and then centrifuged 3000 xg for 10 minutes at room temperature. The solution was desalted with PD-10 column (Amersham Biosciences, GE Healthcare Europe GmbH, Munich, Germany) versus 0.1 M Tris-HCl pH 8.5 and then dialyzed overnight at 4°C in refolding buffer (0.1 M Tris-HCl pH 8.0, 4 mM cystine, 2 mM cysteine). After dialysis the sample was centrifuged 3000 xg for 10 minutes at room temperature, desalted with PD-

10 column to eliminate cystine and cysteine and 10 mM imidazole was added. Recombinant proteins (His-tagged) were affinity purified using $Ni^{2+}$-loaded HiTrap Chelating column. The eluted fractions were loaded on a polyacrylamide gel and stained. Fractions containing the miniprotein of interest were pooled, desalted with PD-10 column to eliminate imidazole, filtrated with Centricon centrifugal filter 10000 MW (Millipore, Billerica, MA) and concentrated with Centricon centrifugal filter 3000 MW (Millipore, Billerica, MA). Polymyxin B (Aerosporin) (Sigma-Aldrich) was added at 10 μg $ml^{-1}$ to eliminate any contaminating endotoxins. This material was used in all subsequent experiments.

## PURIFICATION OF TCMPs FROM TOMATO

Ripe tomatoes were peeled, and after seed removal homogenized with 1 ml $g^{-1}$ of extraction buffer (30 mM Tris-HCl pH 8.2, 50 mM KCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.19% EGTA, 0.5% Tween-20, 0.1% PVPP and protease inhibitors cocktail). The homogenate was centrifuged at 12000 xg for 20 minutes at 4°C and the supernatant was collected. Subsequent centrifugal filtration steps with Centricon 30000 and Centricon 10000 were performed in order to clarify the homogenate. The homogenate were concentrated with Centricon 3000 and centrifuged 12000 xg for 20 min at 4°C to eliminate debries before loading on chromatographic column. TCMP was affinity purified from total extract using either IgG antibodies or carboxypeptidase A (CPA), which were previously immobilised on columns, adopting the AminoLink Plus Immobilization Kit (Thermo Scientific-Pierce Protein Research Products, Rockford, IL).

## NMR SPECTROSCOPY

TCMPs samples (6 mg $ml^{-1}$) were prepared in 20 mM sodium phosphate buffer, pH 7.6, containing 10% $D_2O$. The measurements were performed with a Bruker DRX 500 spectrometer, operating at 500.13 MHz proton Larmor frequency, equipped with a triple resonance probe head, incorporating gradients in the *z*-axis. The measurement temperature was 25°C. Pulse programs from the standard Bruker library (Topspin 1.3) were used. After dissolving the sample, the spectra were run with 128 scans, 16384 data points and relaxation delay of 2 seconds. Presaturation was used to suppress the

residual $^1H_2O$ signal. The data were Fourier-transformed and analysed using standard Bruker software.

## CARBOXYPEPTIDASE INHIBITION ASSAY

Inhibition of carboxypeptidase activity was assayed using 28 nM bovine CPA and 1 mM hippuryl-L-phenylalanine (Sigma-Aldrich, St. Louis, MO) as substrates. Assays were performed according to Hass *et al.* (1981b), monitoring the substrate hydrolysis at 254 nm in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl.

## HUMAN CELL CULTURE

Human Umbilical Vein Endothelial Cells (HUVECs) (Clonetics, Lonza, Basel, Switzerland) were plated on a 25 $cm^2$ flask coated with bovine fibronectin (Sigma-Aldrich, St. Louis, MO). Cells were maintained on endothelial growth media (EGM-2) supplemented with EGM-2 Single Quots (Clonetics, Lonza, Basel, Switzerland) at 37°C and 5% $CO_2$ in a humidified atmosphere.

A431 cells were plated on a 25 $cm^2$ flask. Cells were maintained on RPMI 1640 medium in presence of 10% fetal bovine serum and 50 μg $ml^{-1}$ gentamicin (Sigma-Aldrich, St. Louis, MO) at 37°C and 5% $CO_2$ in a humidified atmosphere. For all applications, HUVEC and A431 cells were used from second to fourth passages.

## IN VITRO CAPILLARY FORMATION OF HUVEC PLATED ON MATRIGEL

The assessment of *in vitro* capillary formation was performed using Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, CA). The wells of 24-well plates were coated with Matrigel under sterile conditions. After gelification of the Matrigel suspension (300 μl per well) for 1 hour at 37°C, $4x10^4$ HUVEC were plated in each well and incubated 24 hours at 37°C and 5% $CO_2$ in a humidified atmosphere in 0.7 ml of EGM-2 complete medium with or without TCMPs (100 and 200 nM) or AG1478 (500 nM). Cells were photographed using a phase contrast microscope (Olympus CK2) coupled with D-100 Nikon digital camera. Tube formation was quantified by measuring the length of tube-like structures with the image-processing software ImageJ

(http://rsb.info.nih.gov/ij/). For each sample the tube length was measured in triplicate, for each replicate 3 fields (2.00x1.33 mm) per well were analysed.

## CELL VIABILITY ASSAYS

Cell viability assay was performed using the WST-8 (MTT) Cell Proliferation Assay Kit (Cayman Chemical Company, Ann Arbor, MI), AnnexinV-FITC Apoptosis Detection kit (Bender MedSystem, Wien) and trypan blue test. For MTT assay, HUVECs either treated or not treated with experimental compounds were seeded, at a density of $1x10^4$ cells/well, in a 96-well plate. After 24 and 48 hours of growth, cell viability was evaluated. Each treatment was performed in triplicate according to manufacturer's instructions.

The percentage of apoptotic cells treated or not with TCMPs was determined using AnnexinV-FITC assay. The method was performed according to the manufacturer's recommendations. HUVECs either treated or not treated with experimental compounds were seeded, at a density of $3x10^4$ cells/well, in a 6-well plate. After 48 hours of growth, cells were stained with annexinV, which binds phosphatidylserine, and then they were stained with propidium, which is a DNA intercalating agent. Stained cells, at least 10000 events, were acquired on a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) and analyzed with the use of FlowJo software (TreeStar, Ashland, USA).

For trypan blue test, HUVECs were seeded, at a density of $48x10^3$ cells/well, in 6-well plates. EGM-2 Single Quots and recombinant peptides were added to the endothelial growth medium. After 48 hours of cultivation, cells were treated with trypsin, collected and resuspended in 0.5 ml of serum-free culture medium. 0.1 ml of 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO) was added to cell suspension and mixed thoroughly. After 5 minutes of incubation at room temperature, trypan blue/cell mixture was applied to a hemocytometer and placed under a Olympus CK2 microscope (100X). The unstained (viable) and blue stained (non-viable) cells were counted.

## CELL PROLIFERATION ASSAYS

Cell proliferation was quantified with 5-ethynyl-2'-deoxyuridine (EdU; Click-iT™ EdU Alexa Fluor Cell Proliferation Assay kit, Invitrogen Ltd, Paisley, UK). EdU is a nucleotide analogue of thymidine that is incorporated into DNA during active DNA synthesis. HUVECs were seeded, at a density of $3x10^4$ cells/well, in a 6-well plate coated with bovine fibronectin in culture medium with or without the recombinant TCMP-2 (200 nM) and 10 μM EdU. After 48 hours cells were detached and washed once with phosphate buffer before being fixed and permeabilized according to the manufacturer's protocol. Stained cells, at least 10000 events, were acquired on a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) and analyzed with the use of FlowJo software (TreeStar, Ashland, USA).

Cell proliferation was also measured by counting the cell numbers. HUVECs were seeded, at a density of $3x10^4$ cells/well, in a 6-well plate coated with bovine fibronectin in culture medium containing the recombinant TCMP-2 (200 nM). After 24, 48, 72 and 96 hours of cultivation, cells were counted in Neubauer chamber using optical microscope Olympus CK2 (100X).

## CELL MIGRATION WOUND ASSAYS

HUVEC were prepared in a single cell suspension. Cells ($1x10^4$) were seeded onto a 24-well culture plate and then cultured to at least 95% confluence. Monolayer cells were washed three times with phosphate-buffered saline. A wound was created in the cells' monolayer with a 10-μl micropipette tip and photographed at a magnification of 100X. Monolayer cells with wound were cultured in serum-free medium for another 24 h with or without stimuli and then photographed at the same position. Cells were treated with different concentrations of TCMPs alone or in combination with 5 ng $ml^{-1}$ EGF (PeproTech, London, UK) or with the specific EGFR inhibitor AG1478 (Calbiochem, Gibbstown, NJ) (500 nM). The area of the cell wounds was calculated using the image-processing software ImageJ. Results are expressed as the mean (± SD).

## SDS-PAGE AND WESTERN BLOTTING ASSAYS

For TCMPs electrophoretic analyses, protein samples were loaded on 15% Tris-Tricine SDS-polyacrylamide gel (PAGE) followed by Coomassie Blue or Silver nitrate staining.

For MAPK signaling pathway analyses, HUVEC and A431 cell lines were treated with different concentrations of TCMPs alone or in combination with 5 ng $ml^{-1}$ EGF or with the specific EGFR inhibitor AG1478 (500 nM). Cells were homogenized using different methods to find the optimal condition for each target protein (EGFR, ERK1/2, Akt): 1) lysed with a hot lysis buffer containing 4% SDS, 20% glycerol, 100 mM Tris-HCl pH 6.8, boiled for 3 minutes, mildly sonicated, centrifuged 1 minute at 16000 xg at room temperature and recovered the supernatant; 2) lysed with CelLytic M (Sigma-Aldrich, St. Louis, MO), transferred in ice for 1 hour, centrifuged 10 min 16000 xg at 4°C then supernatant was recovered; 3) lysed with a lysis buffer containing 2% SDS, 10% glycerol, 50 mM Tris-HCl pH 6.8, 100 mM DTT, mildly sonicated, centrifuged 10 minute at 16000 xg at room temperature and recovered the supernatant; 4) lysed with a cold lysis buffer containing 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 150 mM NaCl, 0.1% SDS, 1% Triton, 10 μg $ml^{-1}$ APMSF, 0.5 μg $ml^{-1}$ leupeptin, 0,7 μg $ml^{-1}$ pepstatin, 50 mM NaF, 1 mM $Na_3VO_4$, 5 mM sodium pyrophosphate, 10 mM sodium-β-glycerolphosphate, 0.5 mM EDTA and 0.5% sodium deoxycholate, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).

Supernatants were separated by 7.5% or 10% polyacrylamide gel electrophoresis (Tris-Glycine SDS-PAGE) and blotted onto a PVDF membrane (Millipore).

The level of total and phosphorylated EGFR was detected using EGFR and phospho-EGFR Tyr1173 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. The level of total and phosphorylated ERK1/2 was detected using ERK1/2 and phospho-ERK1/2 antibodies (Cell Signaling Technology, Danvers, MA), respectively. The level of total and phosphorylated Akt was detected using Akt and phospho-Akt antibodies (Cell Signaling Technology, Danvers, MA), respectively. Blots were developed using chemiluminescent substrate Lite A Blot Plus (Euroclone S.p.A., Siziano, Italy) using horseradish peroxidase (HRP)-conjugated secondary antibodies. The intensity of the chemiluminescence response was measured by scanning the films by GS710

densitometer (Bio-Rad) and processing the image with the Quantity One software Version 4.4 (BioRad). The density of proteins was expressed as absorbance (OD $mm^{-2}$).

## GENE CONSTRUCTS FOR TCMP-1 EXPRESSION IN TOMATO FRUIT

DNA fragments corresponding to the coding sequence for TCMP-1 precursor (77 amino acids; GenBank accession X59282), TCMP-1 mature miniprotein (37 amino acids) and beta-glucuronidase (GUS; as control) were PCR amplified and sub-cloned in pGemT (Promega) and checked by sequencing. Primers used were:

TCMP-1 precursor forward:
5′-CGG*GGTACC*ATGGCACAAAAATTTACT-3′;
TCMP-1 precursor reverse:
5′-TG*GGATCC*TTATCACACGCCTATGGCCATGGC-3′;

TCMP-1 mature miniprotein forward:
5′-CGG*GGTACC*ATGCAGCAATATGATCCAGTTTGT-3′;
TCMP-1 mature miniprotein reverse:
5′-CG*GGATCC*<u>TTATTA</u>AACATAGGGCCCACATGTCCCCGCGAA-3′;

GUS forward:
5′-CGG*GGTACC*ATGTTACGTCCTGTAGAAACC-3′;
GUS reverse:
5′-AA*GGATCC*TCATTGTTTGCCTCCCTGCTGC-3′

The coding region of TCMP-1 precursor and TCMP-1 mature miniprotein were *Kpn*I-*Bam*HI (reported in italics in primer sequences) subcloned in the binary vector pBIN19 downstream to tomato fruit-specific promoter (GenBank accession X13743; Pear *et al*., 1989) and followed by the NOS terminator sequence. The recombinant vectors (pBIN+TCMP-1 precursor, pBINT1tot; pBIN+TCMP-1 mature miniprotein, pBINT1m; pBIN+GUS, pBINGUS) were mobilized into *Agrobacterium tumefaciens* GV2260.

## STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 3.02 software. The mean values ± SE or SD are reported in the figures. Statistical analyses were conducted using a Student's t-test or two-way ANOVA.

# RESULTS

## AMINO ACIDS SEQUENCE ANALYSIS OF TCMPs

Two cystine-knot miniproteins have been identified in tomato. One, hereafter named TCMP-1 is a miniprotein of 37 amino acids in its mature form, highly expressed in the flower (Martineau *et al*., 1991), and the other TCMP-2 is a fruit specific miniprotein of 96 amino acids containing a predicted N-terminal signal peptide (Pear *et al*., 1989).

ClustalW sequence alignment of TCMPs revealed that the mature TCMP-1 miniprotein was highly similar to a cystine-knot miniprotein present in potato (i.d. AAC95130; with a 70% of amino acid identity) which possesses carboxypeptidase inhibitory activity, while the deduced full amino acids sequence of TCMP-2 had high homology with the metallo carboxypeptidase inhibitor PFT2 (i.d. BAA21494; 73% amino acid identity), a member of a small family of cystine-knot miniprotein present in potato tuber (Figure 5).

```
TCMP-1          ------------MAQKFTILFTILLVVIAAQD---------VMAQDATL---TKLFQQY-
PCI             MGRWIHIIFIITMAQKLTILFTILLVVIAAHDNSFYSTKIHVMAQDVVLPTVTKLFQQHA
                            ****:*************:*         *****..*   ******:

TCMP-1          DPVCHKPCSTQDDCSGGTFCQACWRFAGTCGPYVGRAMAIGV
PCI             DPICNKPCKTHDDCSGAWFCQACWNSARTCGPYVGGAMAIGL
                **:*:***.*:*****. ******. * ******* *****:
```

```
TCMP-2          MAAKNSEMKFAIFFVVLLTTTLVDMSGISKMQVMALRDIPPQETLLKMKLLPTNILGLCN
PFT2            MAAKNSEMKFAIFFVVLLTTTLVN------MQVMALRDMPPQETLLKMKLFSSNVLGTCN
                ***********************:      ********:***********:.:*:** **

TCMP-2          EPCSSNSDCIGITLCQFCKEKTDQYGLTYRTCNLLP
PFT2            DYCNTNADCFGITLCPWCKLKKSSSGFTYSECSLLP
                : *.:*:**:***** :** *... *:**  *.***
```

**Figure 5.** Pairwise alignments. Alignments of the deduced amino acid sequences of tomato TCMP-1 miniprotein and the potato metallo carboxypeptidase inhibitor PCI (**upper**) and tomato TCMP-2 miniprotein and metallo carboxypeptidase inhibitor PFT2 (**lower**). Conserved cysteine residues are reported in red. *, residues identical in all sequences; :, for conserved substitutions; ., for semi-conserved substitutions. The sequences of the recombinant proteins are underlined.

Alignment between TCMP-1 and TCMP-2 showed a relative low degree of homology (29% amino acid identity) between the two tomato cystine-knot proteins, however the position of

cystine residues, characteristic of the cystine-knot miniproteins were highly conserved (Figure 6).

```
TCMP-1          MAQK-----FTILFTILLV-------------VIAAQDVMAQDATLTKLFQQYDPV--CH
TCMP-2          MAAKNSEMKFAIFFVVLLTTTLVDMSGISKMQVMALRDIPPQETLLKMKLLPTNILGLCN
                ** *     *:*:*.:**.             *:* :*: .*:: *.  :   : :  *:

TCMP-1          KPCSTQDDCSGGTFCQACWRFAGTCGPYVGRAMAIGV
TCMP-2          EPCSSNSDCIGITLCQFCKEKTDQYGLTYRTCNLLP-
                :***::.** * *:** * . :.  *     .  :
```

**Figure 6.** Pairwise alignments. Alignments of the deduced amino acid sequences of tomato TCMP-1 and TCMP-2 miniproteins. Conserved cysteine residues are reported in red. *, residues identical in all sequences; :, for conserved substitutions; ., for semi-conserved substitutions.

## FLOWER AND FRUIT DEVELOPMENTAL EXPRESSION PATTERN OF TCMP-1 AND TCMP-2

In Angiosperms, once a flower is pollinated and fertilization has successfully taken place, the ovary starts to grow. This is the first visible sign of fruit development (Gillaspy *et al.*, 1993). We divided the flower development in 6 stages (Figure 7).

To investigate the developmental expression patterns of TCMP-1 and TCMP-2 in tomato flower, northern blot was carried out using total RNAs isolated from flower buds at the various stages. The result showed that TCMP-1 was expressed in flower buds from stage 1 (flower buds 6-7 days before anthesis) to 4 (anthesis and 1 day post anthesis). The highest expression level was detected in flower buds 1-3 day before anthesis (stage 3), and the signals then decreased during the flower development. A slight expression of TCMP-1 mRNA is present at the stage of young fruit. The signal of TCMP-2 gradually increased after anthesis from stage 4 to stage 6 (ovary 0.5-1 cm long), and reached its highest level in tomato fruit.

We also detected TCMP-1 and TCMP-2 protein accumulation pattern in the same flower development stages analyzed by northern blot. The results revealed that during the period from stage 3 to stage 6, TCMP-1 protein showed an increasing accumulation, reaching a maximum in stage 5 (4-5 days post anthesis). TCMP-2 protein signal was detectable only in the tomato fruit, where TCMP-1 protein did not appear.

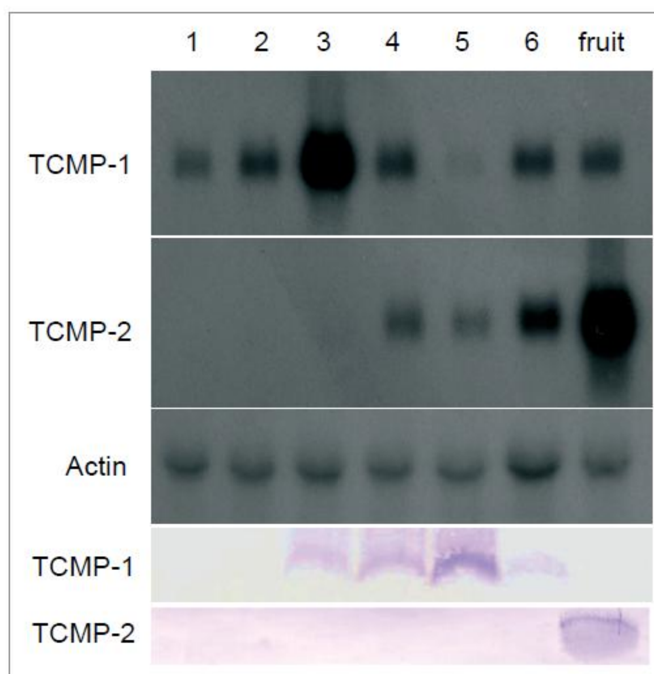


**Figure 7.** Analysis of TCMPs expression in tomato fruit. Northern blot analysis of the TCMPs mRNA at different flower development stages (**upper**); western blot analysis of the TCMP miniproteins at different flower development stages (**lower**). 1: flower buds 6-7 days before anthesis; 2: flower buds 4-5 day before anthesis; 3: 1-3 day before anthesis; 4: anthesis and 1 day post anthesis; 5: 4-5 days post anthesis; 6: ovary 0.5-1 cm long; fruit: tomato red fruit.

## EXPRESSION OF RECOMBINANT TOMATO CYSTINE-KNOT MINIPROTEINS TCMP-1 AND TCMP-2

In order to test the biological activity of the two tomato cystine-knot miniproteins (TCMP-1 i.d. CAA41973; TCMP-2 i.d.AAA34129), they were expressed as recombinant proteins using a bacterial expression system and purified from tomato fruit. The mature 37 amino acid-long TCMP-1 miniprotein and the 44 amino acid-long C-terminal portion of the TCMP-2 miniprotein were expressed in *Escherichia coli* as His-tag fusion proteins (Figure 8).

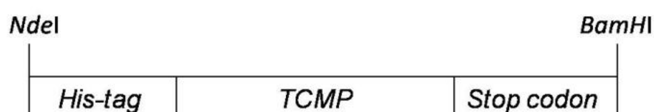


**Figure 8.** Schematic drawing of the construct for the expression of recombinant TCMPs. Restriction endonuclease sites used for the recombinant expression constructs are reported.

Several protocols were tested to optimize the expression level, the solubility and the disulfide bond formation: different *E. coli* strains (BL21, BL21 pLysS, OrigamiB), different induction times (1,3,5,7,24 hours) and concentration of inducer (0.4-0.7-1 mM IPTG) were considered. The recombinant miniproteins were always accumulated as inclusion bodies, irrespective of the cloning strategy applied. The inclusion bodies were extracted and solubilised; then the target proteins were purified with a nickel affinity column and refolded *in vitro*. This procedure were done on column applying a decreasing gradient of denaturant (guanidine hydrochloride 6 M, 30 ml, 0.1 ml $min^{-1}$). However, the $^1H$ NMR analysis indicated the presence of high portion of non well-structured miniproteins (absence of peaks; Figure 9).

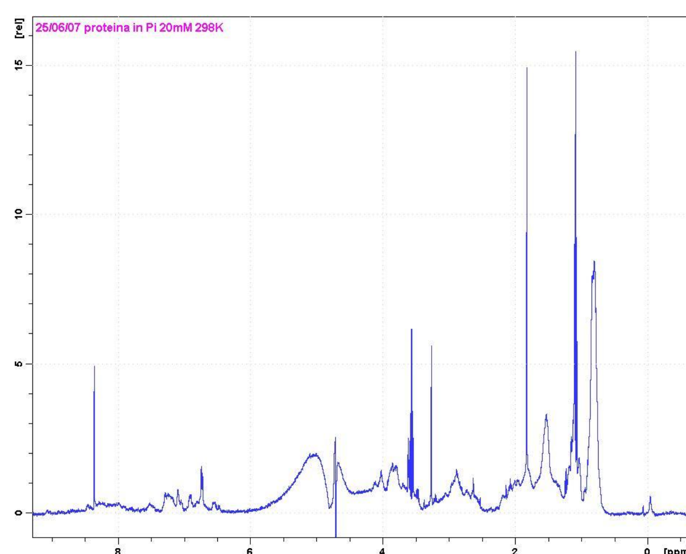


**Figure 9.** $^1H$ NMR spectrum of the TCMP-1. Two milligrams of recombinant TCMP-1 was dissolved in 300 μl of 20 mM phosphate buffer at pH 7.6, containing 10% $D_2O$ and the spectrum was recorded at 25°C in a 500-MHz spectrometer.

In order to improve the solubility of recombinant TCMPs and the yield of the refolding, we tested new strategies of expression and refolding. Three approaches were attempted in order to obtained soluble/folded TCMPs. The first approach consisted of the expression of the target miniproteins as fusion proteins.

The expression of TCMPs as fusion proteins was obtained using the expression vector pET43 1a. This vector was designed to express polypeptide sequences fused with the 495 amino acid long NusA (Nus•Tag™) protein (Figure 10). The NusA sequence has been reported to enhance the solubility of their fusion partners. The Nus•Tag™ vector together with trxB/gor mutant

host strains (OrigamiB strains), facilitate disulfide bond formation in the cytoplasm, which may help maximize the level of soluble, active, properly folded target protein.

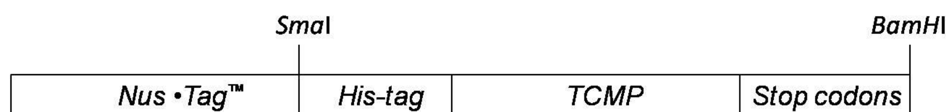


**Figure 10.** Schematic drawing of the construct for the expression of recombinant TCMPs as fusion proteins. Restriction endonuclease sites used for the recombinant expression constructs are reported.

Three *E. coli* strains (BL21, BL21 pLysS, OrigamiB) were transformed with the Nus•Tag™ vector carrying the genes for TCMP. The expression was performed at 37°C, with 2 hours induction and 0.4 mM IPTG; under these conditions BL21 pLysS did not express the recombinant protein, whereas the protein was produced in the BL21 and Origami B backgrounds. The cellular localization of the target proteins expressed in BL21 and Origami strains was verified (Figure 11). Unfortunately, the highest level of protein was still detectable in the inclusion bodies. On the basis of these results the expression of recombinant inhibitors as fusion proteins was abandoned.

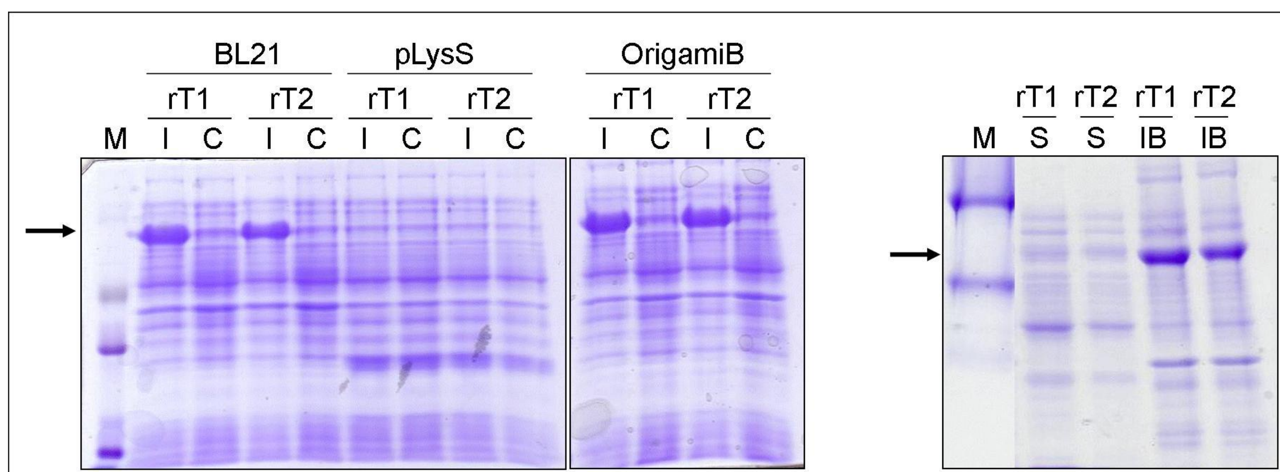


**Figure 11.** Expression of recombinant TCMP-1 and TCMP-2 as fusion proteins. **Left**: SDS-polyacrylamide gel followed by Coomassie blue staining of the expression of target miniproteins assessed by analysis of total cell protein of BL21, BL21pLysS and OrigamiB *E. coli* strains. M: molecular weight marker; I: cells induced with 0.4 mM IPTG; C: control of induction. rT1: recombinant TCMP-1; rT2: recombinant TCMP-2. **Right**: SDS-polyacrylamide gel followed by Coomassie blue staining of the sub cellular localization of fusion TCMP-1 and TCMP-2 proteins in OrigamiB strain. S: cytoplasmic soluble content; IB: inclusion bodies; rT1: recombinant TCMP-1; rT2: recombinant TCMP-2. Arrows indicate the recombinant proteins.

An alternative strategy to obtain soluble tomato cystine-knot inhibitors was to use a vector that enables the export of the protein in the periplasm, which is a more favourable environment for folding and disulfide bond formation. For this purpose the pET12b vector carrying a signal sequence for translocation into the periplasmic space was used. At the amino terminal of the protein the enterokinase cleavage site was integrated (Figure 12).

**Figure 12.** Schematic drawing of the construct for the expression of recombinant TCMP-2 in the periplasmic space. Restriction endonuclease sites used for the recombinant expression construct are reported.

Three *E. coli* strains (BL21, BL21 pLysS, OrigamiB) were transformed with the pET12b vector carrying the genes for TCMP-2. The expression was performed at 37°C, 3 hours induction and 0.4 mM IPTG. Only the BL21 pLysS strain was able to express the target protein (Figure 13, left). However, verification of the sub cellular localization of the target protein and purification of the recombinant protein was not easy with host strains containing pLysS, because T7 lysozyme damages the cell wall during the manipulation. Western blot analysis of the TCMP-2 localisation revealed the recombinant protein in all presumed compartments (Figure 13 right) and it was not possible to well define the amount of protein present in the periplasm.

We analyzed the medium as well (Figure 13 right, lane 4), because Molina and collegues found that using a vector for the export of potato carboxypeptidase inhibitor into the periplasmic space, recombinant PCI was found almost exclusively in the culture medium (Molina *et al*., 1992). In our experimental conditions, the concentration of the recombinant protein in the culture medium was rather low. Also the approach of expression of recombinant inhibitors in the periplasmic space was abandoned, because the purification from the external medium would be too laborious.

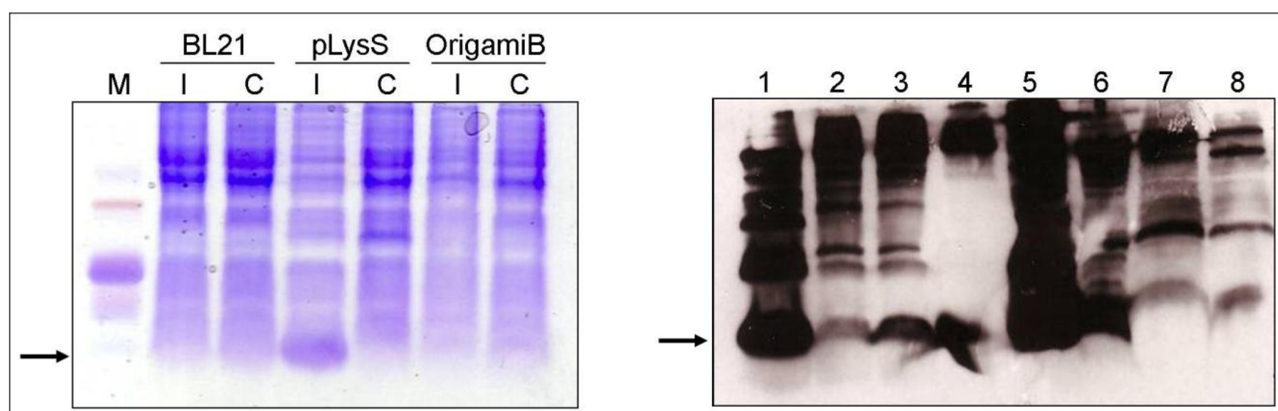


**Figure 13.** Expression of recombinant TCMP-2 in periplasmic space. **Left**: SDS-polyacrylamide gel followed by Coomassie blue staining of the total cell protein. M: molecular weight marker; I: cells induced with 0.4 mM IPTG; C: control of induction. **Right**: western blot of steps during the sub cellular localization analysis of expressed proteins in BL21pLysS *E. coli* strain. 1: whole cell lysate; 2: content of the presumed periplasmic space; 3: waste of the purification step of the presumed periplasmic space; 4: cell culture medium; 5: denatured inclusion bodies; 6: cytoplasmic soluble extract; 7-8: wash steps of the cytoplasmic content isolation. Arrows indicate the recombinant protein.

The third approach to increase the yield of the recombinant proteins was the application of a new refolding protocol. Bronsoms *et al*. demonstrated that potato carboxypeptidase inhibitor can correctly refold *in vitro* with kinetics and efficiencies depending on the redox condition used, so we decided to apply the same refolding protocol (Bronsoms *et al*., 2003). After a 5 hour-long solubilisation of inclusion bodies, the denaturant was eliminated with a desalting column and the refolding was done with an overnight 4°C dialysis with a buffer containing 4 mM cysteine and 2 mM cystine. The day after, the sample was centrifuged and desalted to eliminate both cysteine and cystine. Target proteins were purified by means of affinity chromatography. Analysis of miniproteins on a SDS-polyacrylamide gel followed by silver nitrate staining showed the presence of numerous bands of higher molecular weight. TCMP were then isolated with an ultrafiltration step using a membrane with cut off 10 kDa. The sample recovered in the filtrate was analysed on a SDS-polyacrylamide gel followed by Coomassie blue or silver nitrate staining and it showed an unique band of the correct apparent molecular weight (Figure 14).

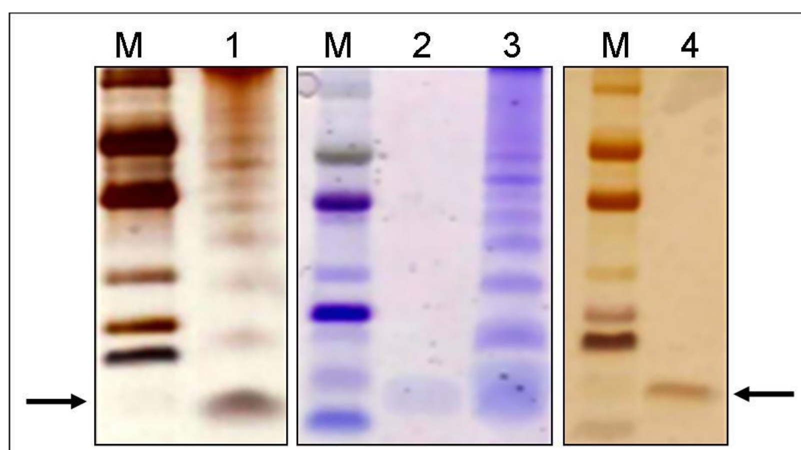


**Figure 14.** Representative SDS-PAGE of recombinant TCMPs after the refolding protocol with redox agents. SDS-polyacrylamide gels followed by silver nitrate staining or Coomassie blue staining showing the purity of recombinant TCMPs before and after the ultrafiltration step. M: molecular weight marker. Lane 1 is the affinity chromatography eluate pool; lanes 2 and 4 show the presence of TCMPs in the sample passed through the membrane 10000 MW stained with Coomassie blue or silver nitrate, respectively; lane 3 represents the retained macrosolutes above the membrane 10000 MW. Arrows indicate the bands corresponding to TCMPs.

$^1$H NMR analysis of TCMP-2 refolded with this new strategy showed a better-structured miniprotein, even if it was not possible to establish if it corresponds to its native conformation.

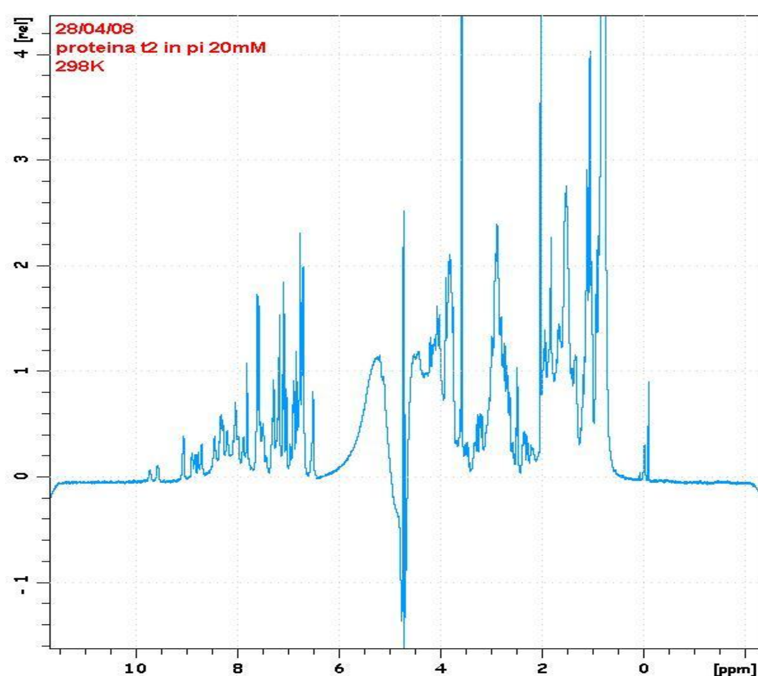


**Figure 15.** $^1$H NMR spectrum of the TCMP-2. Two milligrams of refolded TCMP-2 was dissolved in 300 µl of 20 mM phosphate buffer at pH 7.6, containing 10% $D_2O$ and the spectrum was recorded at 25°C in a 500-MHz spectrometer.

## PURIFICATION OF TCMPs FROM TOMATO FRUIT

TCMP was also extracted from mature tomato fruits in a two-step procedure including homogenization and affinity chromatography against either polyclonal antibody raised against TCMP-1 recombinant protein or carboxypeptidase A (Hass and Ryan, 1980). The purified miniproteins showed identical electrophoretic mobility irrespective of the purification method (data not shown). The analysis of the different fractions obtained during immunopurification procedure is shown in Figure 16.

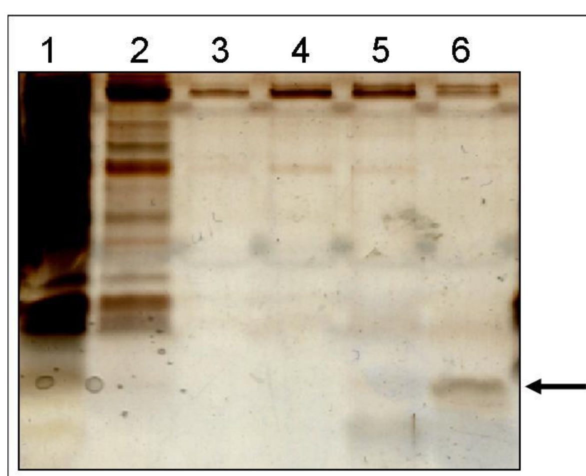


**Figure 16.** Immunoaffinity purification step of TCMPs from tomato fruit. Proteins were resolved by SDS-polyacrylamide gel followed by silver nitrate staining. Lane 1 represent crude tomato fruit homogenate; lanes 2-5 are wash fractions; lane 6 represent affinity chromatography eluate pool (pure protein preparation). The arrow indicates the band corresponding to the tomato native TCMPs.

The apparent molecular masses of the electrophoretised recombinant miniprotein TCMP-1 and TCMP-2 and the TCMPs extracted from tomato fruits were consistent with the electrophoretic migration of native miniproteins present in tomato fruit crude extract (Figure 17).

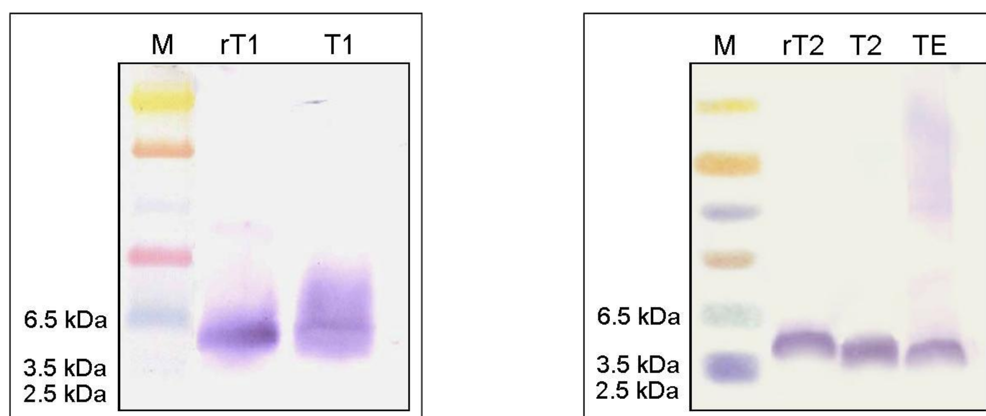


**Figure 17.** Western blot analysis of recombinant TCMP miniproteins. **Panel left**: TCMP-1. M: molecular weight marker; rT1: purified recombinant TCMP-1; T1: flower buds extract. **Panel right**: TCMP-2. M: molecular weight marker; rT2: purified recombinant TCMP-2; T2: TCMP miniproteins purified from tomato fruit; TE: tomato fruit crude extract.

## INHIBITION OF CARBOXYPEPTIDASE A BY TCMP-2

Potato carboxypeptidase inhibitor (PCI) competitively inhibits several metallo carboxypeptidases with a *K*i in the nanomolar range (Hass *et al*., 1979a). A similar inhibitory activity towards carboxypeptidase A (CPAase) was proved for tomato TCMP-1 extracted from wound-induced leaves (Díez-Díaz *et al*., 2004). Potato GM-7 type miniproteins have been annotated as metallo carboxypeptidase inhibitors on the basis of their sequence similarity with PCI and TCMP-1; nevertheless, their inhibitory activity has not been proved. We observed that the recombinant TCMP-2 miniprotein possesses inhibitory activity against the bovine carboxypeptidase A (Figure 18).

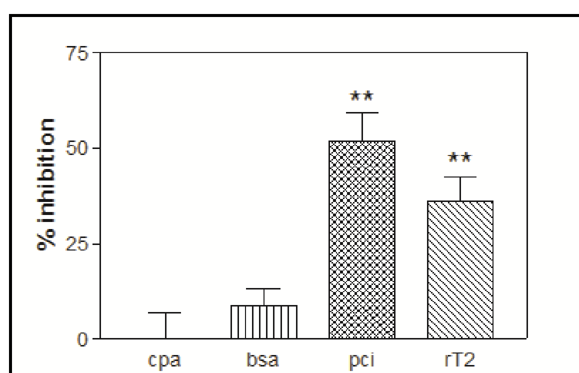


**Figure 18.** Inhibitory effect of TCMP-2 on bovine carboxypeptidase A (CPA) activity. PCI: potato carboxypeptidase inhibitor, rT2: 200 nM recombinant TCMP-2; BSA: bovine serum albumin. (**$P<0.01$ versus CPA). Bars indicate SD.

## INHIBITION OF IN VITRO ANGIOGENESIS BY TCMPs

Potato PCI is an antagonist analogue of EGF able to inhibit the growth of pancreatic adenocarcinoma cells (Blanco-Aparicio *et al.*, 1998). On the basis of the structural homology between TCMP miniproteins and potato PCI, current knowledge suggests that these miniproteins may display a spectrum of biological effects in human cells related to their predictable activity as EGF antagonist. EGF and its receptor (EGFR) are involved in many aspects of the development of carcinoma including cell growth and vascularisation. EGFR antagonists and blockers can inhibit neo-vascularisation during tumor progression (Michaelis *et al.*, 2003). To test the hypothesis that TCMP miniproteins can act as anti-angiogenic compounds, we investigated the effects of TCMP miniproteins, both natural and recombinants, on *in vitro* angiogenesis using Matrigel matrix. The EGFR inhibitor AG1478 was tested as a positive control. To exclude side effects on capillary tube formation caused by bacterial lipopolysaccharides (LPS), recombinant protein preparations were treated with 10 μg $ml^{-1}$ polymyxin B, an antibiotic which binds to and neutralizes LPS (Cardoso *et al.*, 2007). The selective epidermal growth factor (EGF) receptor blocker AG1478 exhibited a strong inhibitory effect on tube formation at 500 nM, indicating that EGF signaling pathway activation is a crucial event in capillary network formation. HUVEC and progenitor endothelial cells treated with nanomolar concentrations of TCMP miniproteins were not able to form a complete vascular network within 24 hours as compared with control (Figure 19).

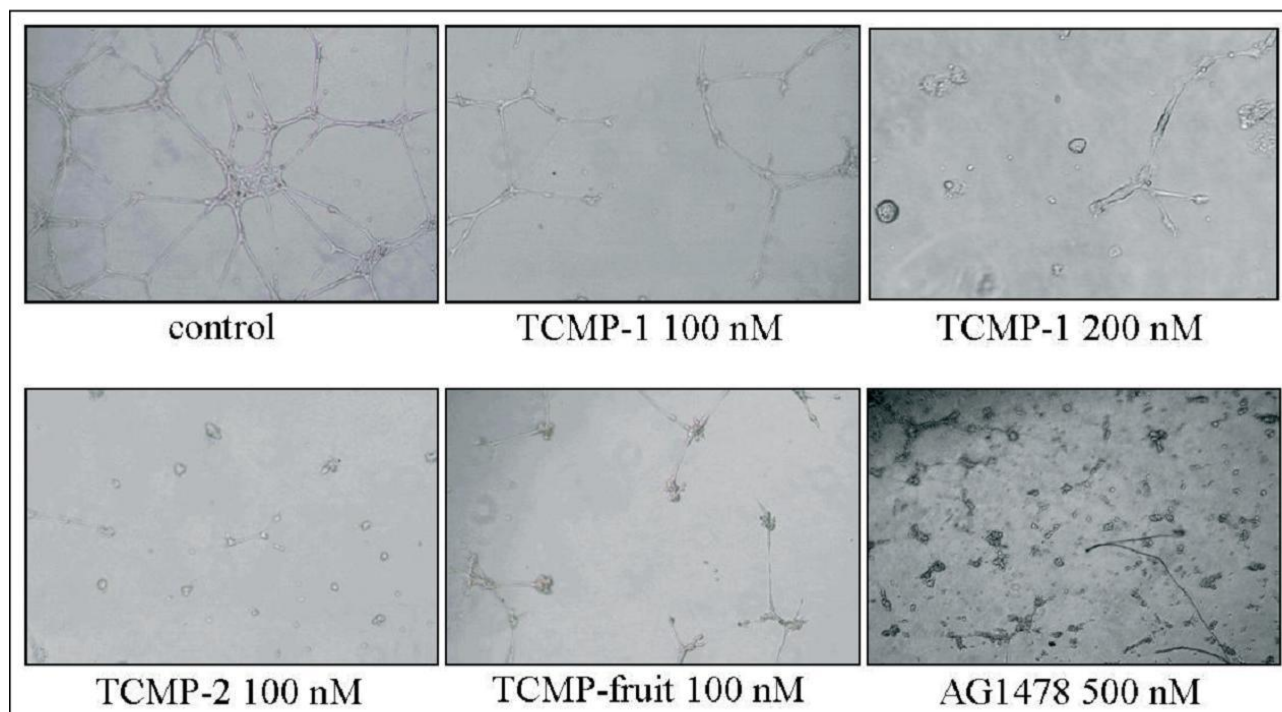


**Figure 19.** Effects of tomato cystine-knot miniproteins on in vitro angiogenesis. HUVEC grown on growth factor-reduced Matrigel were treated with recombinant TCMPs and TCMPs purified from mature tomato fruits. Endothelial cells were observed 24 hours after treatment and results were recorded digitally.

Quantitative analysis of vascular network length was carried out to characterize the anti-angiogenic effect of TCMP miniproteins (Figure 20). We found a significant reduction of tube length in the presence of TCMP miniproteins at all the concentrations tested (20, 50 and 100 nM). At the highest concentration (100 nM) the vascular network length was reduced by 64% (TCMP-1) and 86% (TCMP-2), respectively as compared with that measured in untreated HUVECs. Native TCMP miniprotein, extracted from mature tomato fruit, depressed tube formation as well, and the effect was comparable with that of the recombinant miniproteins.

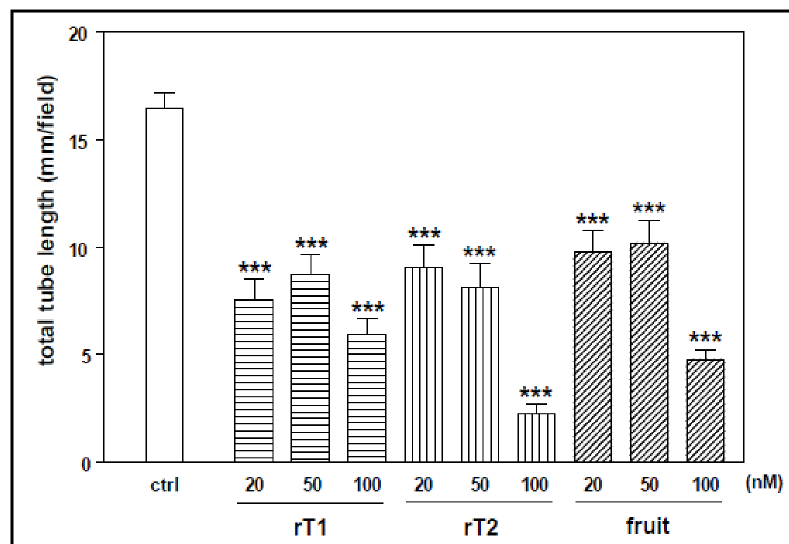


**Figure 20.** Quantitative analysis of tube length formation. The total tube length of a 2.66 $mm^2$ field was measured, for each treatment 3 fields per well were randomly chosen. The average tube length was calculated analyzing 3 wells per treatment (***P<0.001 versus control). ctrl: control; rT1: purified recombinant TCMP-1; rT2: purified recombinant TCMP-2; fruit: TCMP miniproteins purified from tomato fruit. Bars indicate SD.

The formation of vascular network was not affected when HUVEC were treated with peptides corresponding to the N-terminal (17 amino acids in length) or the C-terminal (20 amino acids in length) regions of TCMP-1 (Figure 21), suggesting that the two fragments did not possess an intrinsic anti-angiogenic activity and excluding aspecific affects of the miniproteins.

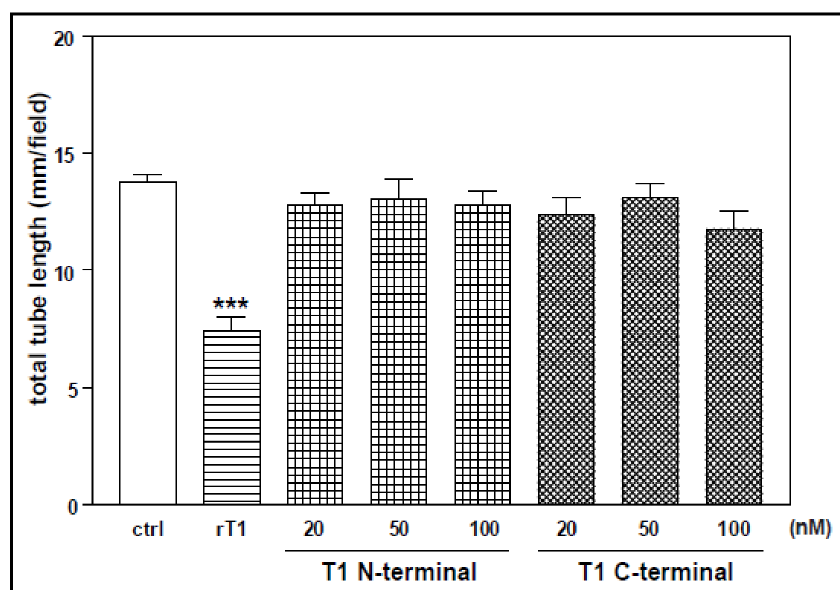


**Figure 21.** Quantitative analysis of tube length formation. The total tube length of a 2.66 $mm^2$ field was measured, for each treatment 3 fields per well were randomly chosen. The average tube length was calculated analyzing 3 wells per treatment (***P<0.001 versus control). ctrl: control; rT1: purified recombinant TCMP-1; T1 N-terminal and T1 C-terminal indicate the treatment with peptides corresponding to the N-terminal (17 amino acids in length) or the C-terminal (20 amino acids in length) regions of TCMP-1, respectively. Bars indicate SD.

These results indicate that both recombinant and native TCMP miniproteins exhibit anti-angiogenic effects at concentrations in the nanomolar range.

# EFFECTS OF TCMP MINIPROTEINS ON ENDOTHELIAL CELLS VIABILITY

Angiogenesis is a multi-step process that involves a phase of active proliferation of endothelial cells. In order to test the effects of TCMP miniproteins on endothelial cell vitality, we treated HUVEC with TCMP miniproteins at the concentration of 200 nM and we measured cell viability after 24 and 48 hours of culture by MTT assay (WST-8). The recombinant TCMP-1 miniprotein does not induce any toxic effect on HUVEC (Figure 22). A modest reduction on vital cell number (5%) was detected with recombinant TCMP-2 after 24 hours of treatment and with TCMP purified from mature tomato fruit after 48 hours of treatment. The two synthetic peptides, corresponding to the first 17 N-terminal amino acids and to the last 20 C-terminal amino acids of TCMP-1, respectively, display a similar modest effect on HUVEC vitality.

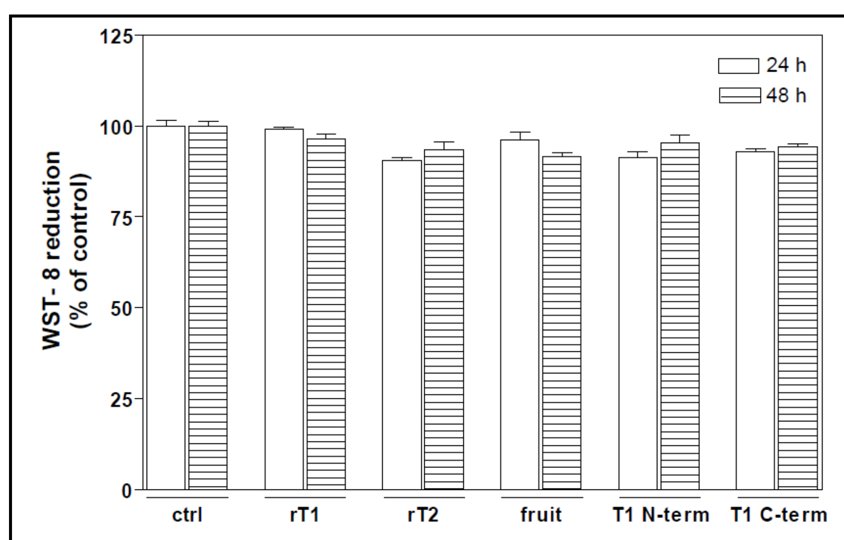


**Figure 22.** Effects of tomato cystine-knot miniproteins on HUVEC viability: MTT assay. The average percentage of vital cells after 24 and 48 hours of treatment is reported. Ctrl: control; rT1: purified recombinant TCMP-1; rT2: purified recombinant TCMP-2; fruit: TCMP miniproteins purified from tomato fruit; T1 N-term and T1 C-term indicate the treatment with peptides corresponding to the N-terminal (17 amino acids in length) or the C-terminal (20 amino acids in length) regions of TCMP-1, respectively. Bars indicate SD.

The effects of TCMP miniproteins on HUVEC viability was also assayed using the trypan blue exclusion method (data not shown) and the annexin V-propidium iodide assay (Figure 23). Annexin V exhibits anti-phospholipase activity and binds to phosphatidylserine, which was

exposed at cell surface during the execution of apoptosis. Annexin-FITC labelling allows simple direct detection by flow cytometer analysis. Counterstaining by propidium iodide, a DNA intercalating agent, allows the discrimination of apoptotic cells.

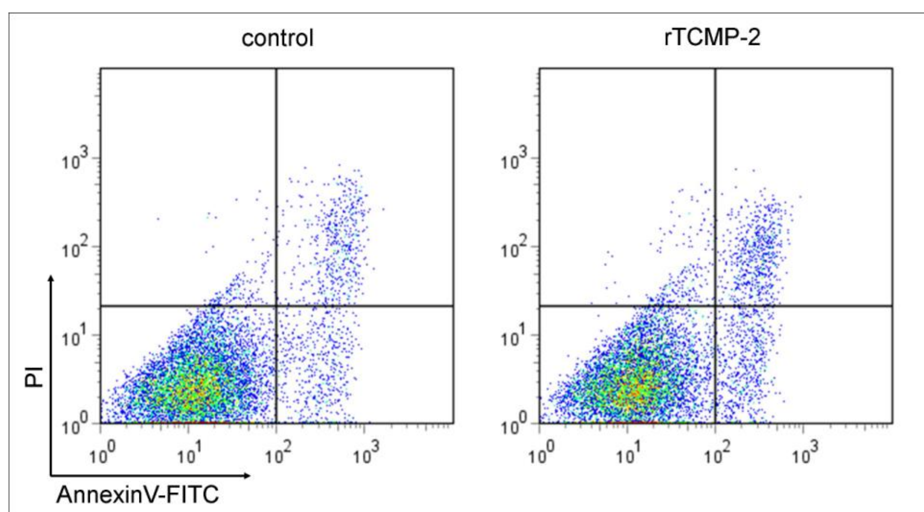


**Figure 23.** Dual parameter dot plot of HUVEC stained with annexin V (AnnexinV-FITC) and propidium iodide (PI). Cells were treated for 48 hours with 200 nM TCMP-2. Left: viability plot of HUVEC without treatment as control; right: viability plot of HUVEC after treatment with recombinant TCMP-2.

The data obtained with these assays confirmed the absence of significant toxic effects of TCMP miniproteins on HUVEC (Figure 24).

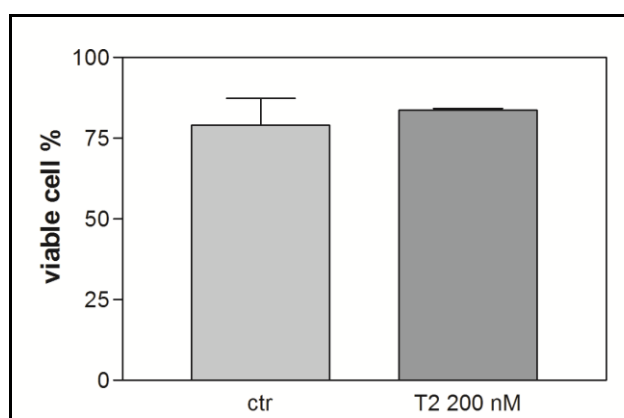


**Figure 24.** Annexin assay. Ctr is the percentage of viable HUVEC without treatment; T2 200 nM represents the percentage of viable HUVEC after treatment with 200 nM of recombinant TCMP-2. Bars indicate SD.

## EFFECTS OF TCMP MINIPROTEINS ON ENDOTHELIAL CELLS PROLIFERATION

Cell proliferation was assayed with the incorporation into DNA during active DNA synthesis of EdU, a nucleoside analogue of thymidine (Figure 25). Detection is based on a click reaction (Breinbauer and Köhn, 2003; Wang *et al.*, 2003), a copper catalyzed covalent reaction between an azide and an alkyne. The EdU contains the alkyne, while the dye contains the azide. Standard flow cytometry methods are used for determining the percentage of cells in the population that are in S-phase. The assay has demonstrated that the treatment with TCMP-2 for 48 hours does not affect the proliferation capability of HUVEC cells.

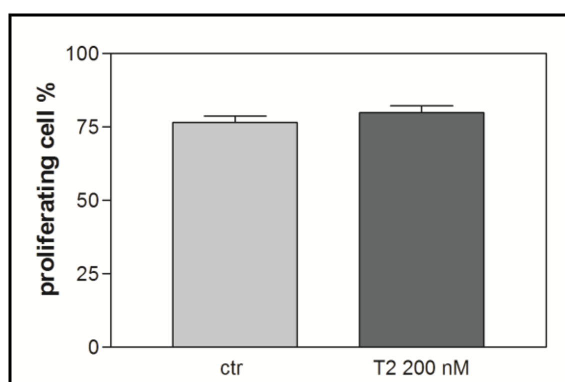


**Figure 25.** EdU Proliferation assay. Ctr represents the percentage of proliferating HUVEC without treatment; T2 200 nM is the percentage of HUVEC after treatment with 200 nM of recombinant TCMP-2. Bars indicate SD.

As an independent control, the growth of HUVEC cells was followed up to 96 hours in the presence of TCMP-2 at a concentration of 200 nM. The treatment did not affect cell proliferation (Figure 26).

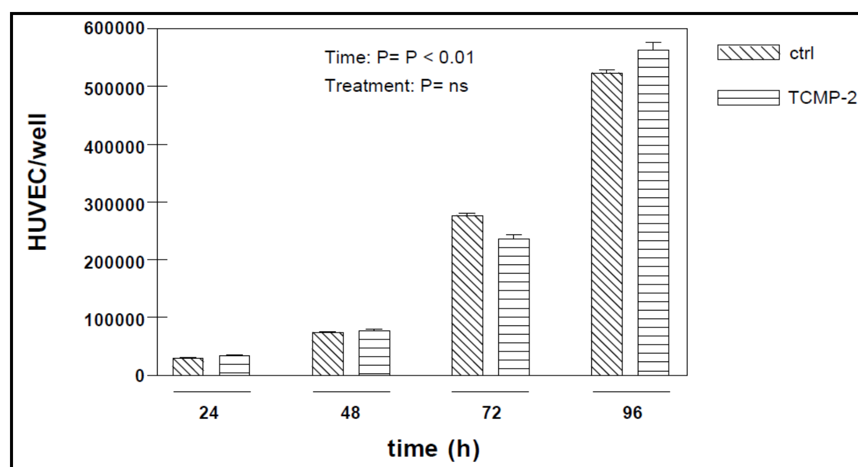


**Figure 26.** Cell count. Number of HUVEC per well 24, 48, 72, 96 hours after treatment with 200 nM TCMP-2. Bars indicate SE.

These data indicate that TCMP miniproteins do not significantly affect HUVEC proliferation, suggesting that the anti-angiogenic effect does not depend on the inhibition of cell proliferation or viability.

## EFFECT OF TCMPs ON EGFR SIGNALING PATHWAY

EGFR signal transduction cascade is implicated in angiogenesis; oncogenic EGFR contributes to angiogenic phenotype of cancer cells (Al-Nedawi *et al.*, 2009). The cystine-knot potato carboxypeptidase inhibitor at concentrations of about 10 μM was reported to interfere with EGFR activation through inhibition of receptor transphosphorylation induced by EGF (Sitjà-Arnau *et al.*, 2005). To investigate the mechanism underlying the anti-angiogenic effect displayed by TCMPs, we monitored the phosphorylation state of EGFR in the presence of the same TCMPs concentrations used in the angiogenesis assay. One fundamental signaling pathway downstream EGFR activation is mitogen activated protein kinase (MAPK) cascade. Therefore we also examined the phosphorylation state of ERK1/2, one of the main effector proteins downstream to EGFR, to determine whether TCMP affect the MAPK pathway.

EGFR-overexpressing A431 cells were incubated for 30 minutes with nanomolar concentrations of PCI, recombinant TCMP-2 and AG1478 alone or 30 minutes with nanomolar concentrations of PCI, recombinant TCMP-2 and AG1478 and 5 or 15 minutes with EGF to test their activity on EGFR phosphorylation or on ERK1/2 and Akt phosphorylation, respectively. The EGFR specific inhibitor AG1478 at a concentration of 500 nM completely blocked the receptor transphosphorylation induced by EGF. PCI at the concentration of 500 nM was not able to block the autophosphorylation of EGF-stimulated EGFR. Similarly, recombinant TCMP-2 (100-500 nM) did not depress EGFR activation. Moreover, cells treated with TCMP-2 concentration greater than 100 nM or with PCI showed autophosphorylation of the EGF receptor (Figure 27).

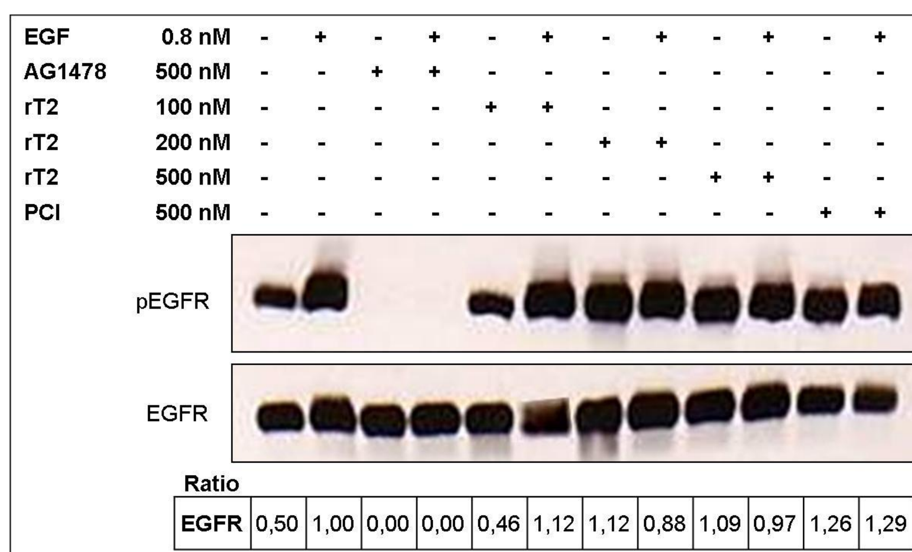


**Figure 27**. Western blot analysis of phospho-EGFR (pEGFR) performed on A431 cells treated with different concentrations of TCMP-2 (rT2). The ratio of the densities (phosphorylated form/total protein) is shown in the lower table. The experiments were performed at least twice and a representative example is shown.

Both PCI and TCMP-2 had only minor effects on ERK1/2 phosphorylation when they were supplied in combination with EGF, but they showed an inhibitory activity when used alone. This inhibitory effect was more evident on ERK1 (Figure 28).

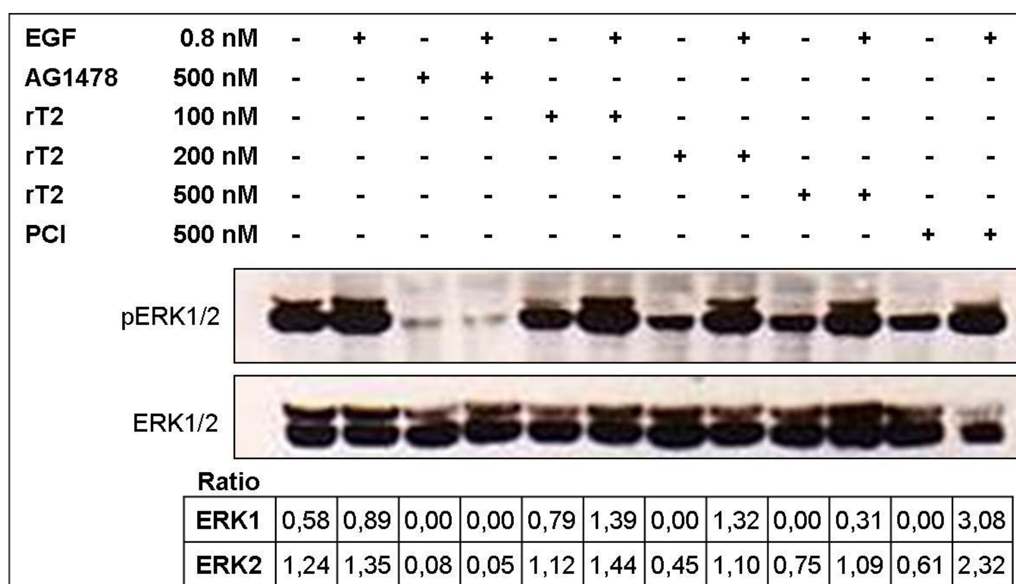


**Figure 28.** Western blot analysis of phospho-ERK1/2 (pERK1/2) performed on A431 cells treated with different concentrations of TCMP-2 (rT2). The ratios of the densities (phosphorylated form/total protein) are shown in the lower table. The experiments were performed at least twice and a representative example is shown.

Every tested concentration of TCMP-2 and PCI had very little or no effect on Akt phosphorylation when A431 cells were inoculated with EGF (Figure 29).

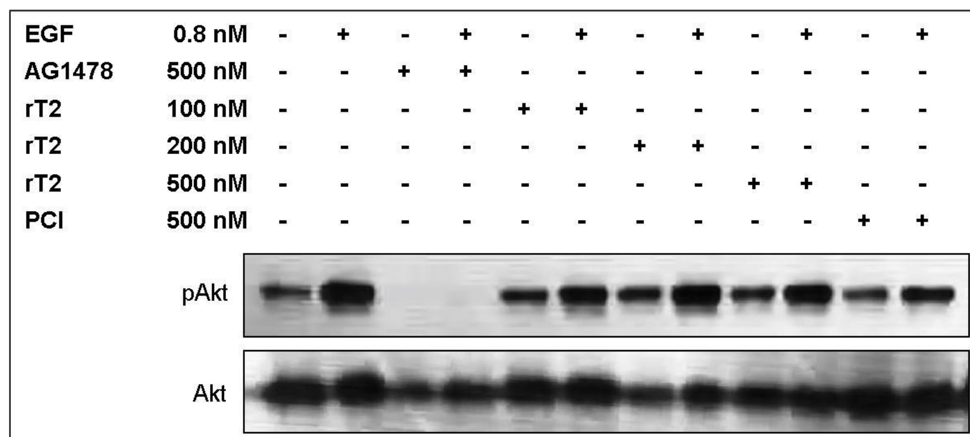


**Figure 29.** Western blot analysis of phospho-Akt (pAkt) performed on A431 cells treated with different concentrations of TCMP-2 (rT2). The experiments were performed at least twice and a representative example is shown.

These data suggest that TCMP-2 and PCI in the nanomolar range interfere with MAPK signaling pathway affecting ERK1/2 activation and not Akt phosphorylation, and this effect is not the consequence of an inhibitory action on EGFR phosphorylation.

## TCMP MINIPROTEINS INHIBIT ERK1/2 PHOSPHORYLATION

To test whether TCMP miniproteins can interfere with MAPK pathway of endothelial cells, EGF-stimulated HUVEC cells were treated with nanomolar concentrations of recombinant TCMPs and the phosphorylation level of ERK1/2 was examined. The results show that phospho-ERK1/2 decreased after treatment with recombinant TCMP miniproteins at 100 nM (Figure 30, panel left). The same effect was obtained after treating HUVEC with PCI at 100 nM. The effect produced by TCMPs on phospho-ERK1/2 level was weaker than that observed in HUVEC treated with the EGFR specific inhibitor AG1478 at 500 nM. The inhibitory effect of TCMPs was particularly evident against ERK1.

TCMP extracted from mature fruits showed an inhibitory effect on ERK1/2 phosphorylation similar to that observed with recombinant proteins (Figure 30, panel right).

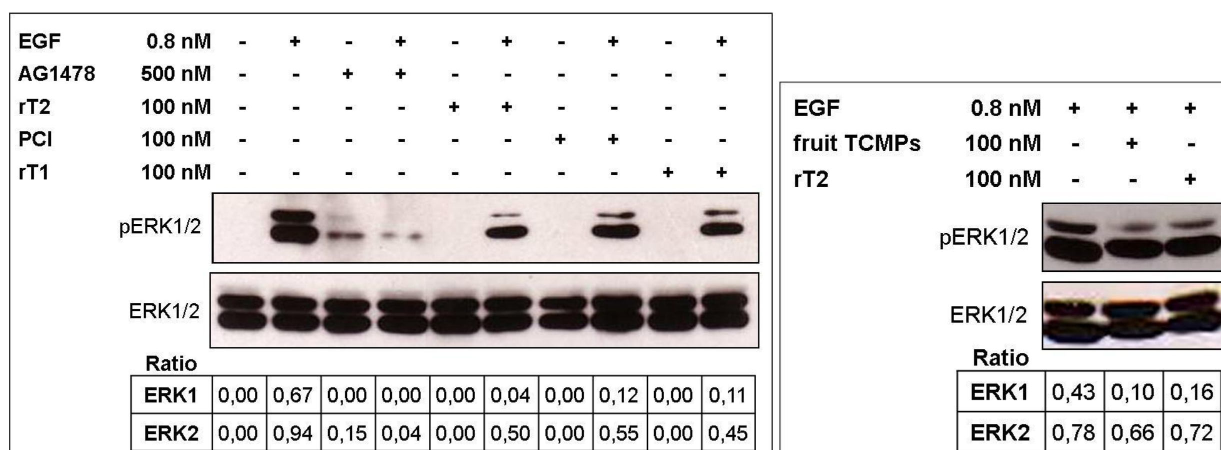


**Figure 30.** Western blot analysis of p-ERK1/2 performed on HUVEC treated with TCMPs. **Panel left:** HUVEC treated with recombinant TCMP-1 (rT1) and TCMP-2 (rT2); **Panel right:** HUVEC treated with TCMPs purified from tomato fruit (fruit TCMPs). The ratio of the densities (phosphorylated form/protein) are shown in the lower panel of each western blot. The experiments were performed at least twice and a representative example is shown.

These data suggest that the anti-angiogenic effect exerted by TCMPs is associated to the alteration of MAPK signaling pathway.

Only the non-phosphorylated form of Akt was detected in HUVEC despite the use of different lysis buffers and different amount of cell lysate loaded on SDS-PAGE. pEGFR, EGFR gave no signals. Although evidence suggests a direct effect of EGF on endothelial cells, the expression and function of EGFR in this cell type is still debated. Several studies have failed to demonstrate expression of EGFR in HUVEC (Hirata *et al*., 2002; Amin *et al*., 2006); however, Sini and colleagues have demonstrated that stimulation of HUVEC with EGF led to an increase of EGFR phosphorylation and ERK1/2 activation (Sini *et al*., 2005). Some authors concluded that heterogeneous results on the expression of EGFR in endothelial cells might be relate to differences between individuals as well as to cell culture variability. However, activation of EGFR signaling in endothelial cells could occur in specific physiological situation.

## TCMP-1 EXPRESSION IN TOMATO FRUIT

In order to increase the TCMPs content of tomato fruit, we planned to stably transform Micro-Tom plants with a construct containing the *TCMP-1* gene under the control of a tomato fruit specific promoter.

Micro-Tom is a miniature dwarf tomato cultivar that has several unique features, such as a small size that enables it to grow at a high density, seed setting under fluorescent light and a short life cycle that allows for mature fruit to be harvested within 70–90 days after sowing.

*TCMP-1* encodes for a 643 base-long mRNA which produces a putative protein of 77 amino acid residues (Figure 31). On the basis of the protein sequence (CAA41973.1), the molecular mass is estimated to be approximately 8.3 kDa with an isoelectric point of about 6.5. TCMP-1 protein is predicted to have a N-terminal 37 residue-long signal peptide, which is excised (Figure 31) and most likely targets the mature protein towards the secretory pathway, consistent with what has been observed for other proteins containing the same hydrophobic signal sequence associated with transport of the nascent polypeptide across the rough endoplasmic reticulum (von Heijne, 1983). TCMP-1 protein is predicted also to have a C-terminal 8 residue-long extension for vacuolar sorting (Figure 31).

> X59282 *Solanum lycopersicum* mRNA *for TCMP-1 gene*<br>
ATTATTATTACCATGGCACAAAAATTTACTATCCTTTTCACCATTCTCCTTGTGGTTATTGC<br>
TGCTCAAGATGTGATGGCACAAGATGCAACTCTGACGAAACTTTTTCAGCAATATGATCCAG<br>
TTTGTCACAAACCTTGCTCAACACAAGACGATTGTTCTGGTGGTACGTTCTGTCAGGCCTGT<br>
TGGAGGTTCGCGGGGACATGTGGGCCCTATGTTGGGCGCGCCATGGCCATAGGCGTGTGATT<br>
ACAATTTCGTTGTTCTTCTTTTTCGACTTTTTAATCCCAAGTGAATAAAGTCTAATTCGAAA<br>
AAGAAGAAAAAAGTATCTATGTCTGAGTTATATGTTTTGTGGCTAATAAGAAATCGACTATG<br>
CTTGTTGATTTGATAAAAATTATGTCATTAGGGTGTGATATGTAATCATCAAATTAAATAAA<br>
AATCATCGCATTGTGTGTGC

> CAA41973.1 TCMP-1<br>
MAQKFTILFTILLVVIAAQDVMAQDATLTKLFQQYDPVCHKPCSTQDDCSGGTFCQACWRFA<br>
GTCGPYVGRAMAIGV

> TCMP-1<br>
N-**MAQKFTILFTILLVVIAAQDVMAQDATLTKLF**QQYDPVCHKPCSTQDDCSGGTFCQACWR<br>
FAGTCGPYV**GRAMAIGV**-C

**Figure 31**. TCMP-1 sequences. **Upper panel**: nucleotide sequence of the mRNA encoded by TCMP-1 gene. **Middle panel**: amino acid sequence of TCMP-1 protein. **Lower panel**: amino acid sequence of TCMP-1 with in bold N-terminal signal peptide and C-terminal extension.

Two distinct constructs were done to express in tomato fruit either the precursor (pBINT1tot) or the mature form of TCMP-1 (pBINT1m). The constructs were placed under the control of TCMP-2 promoter which has been demonstrated to be fruit specific (Pear *et al*., 1989). As control of the promoter activity, a third construct containing the coding region of the GUS gene under the control of the TCMP-2 promoter was created (pBINGUS).

pBIN19 carries the *lacZ'* gene, the kanamycin-resistance gene (*kan*$^R$), an *E. coli* origin of replication, and the two boundary sequences from the T-DNA region of the Ti plasmid. These two boundary sequences recombine with plant chromosomal DNA, inserting the segment of DNA between them into the plant DNA. The orientation of the boundary sequence in pBIN19 means that the *lacZ'* and *kan*$^R$ genes, as well as any new DNA ligated into the restriction sites within *lacZ'*, are transferred to the plant DNA (Figure 32).

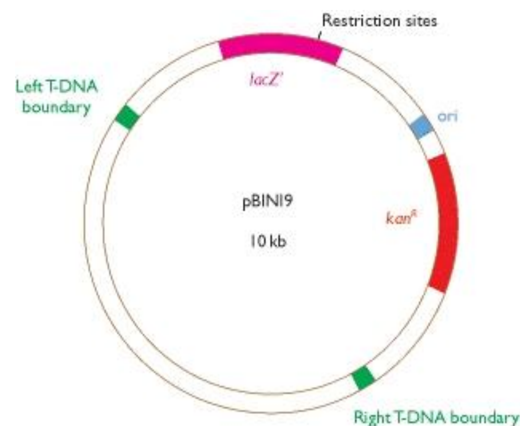


**Figure 32.** pBIN19 map.

pBIN is an example of a shuttle vector, recombinant molecules being constructed in *E. coli*, using the *lacZ'* selection system, before transfer to *Agrobacterium tumefaciens* and hence to the plant. Subsequently, recombinant plant cells are selected by plating onto kanamycin agar, and then regenerated into whole plants.

TCMP-1 and GUS coding region were amplified using PCR adding the *Kpn*I restriction site at the 5' end and the *Bam*HI restriction site at the 3' end. The tomato fruit-specific promoter sequence was also amplified by PCR adding the *Eco*RI and *Kpn*I restriction sites at the 5' and 3' end, respectively. The resulting amplicons were cloned into linearized plasmid pGemT and checked by sequencing. The recombinant genes and promoter were subsequently digested with restriction enzymes *Eco*RI, *Kpn*I and *Bam*HI and introduced into the binary vector pBIN19 carrying the NOS terminator as indicated in Figure 33a. The three recombinant plasmids where checked by DNA sequencing and introduced in *Agrobacterium tumefaciens* GC2260. The introduction of the recombinant binary vectors in *A. tumefaciens* was verified by PCR (Figure 33b).

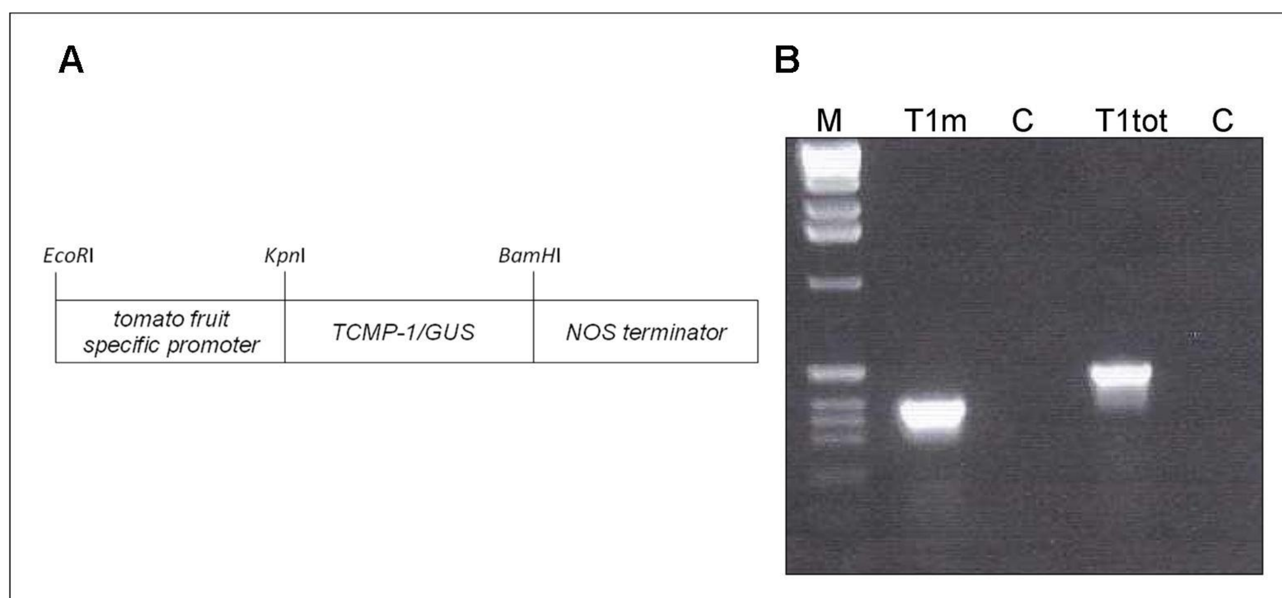


**Figure 33. A:** Scheme of the chimeric genes created for tomato transformation. **B**: Agarose gel electrophoresis of PCR products obtained from plasmid pBINT1m and pBINT1tot extracted from *Agrobacterium tumefaciens* GC2260. M: molecular weight marker; T1m: TCMP-1 mature sequence; T1tot: TCMP-1 precursor sequence; C: PCR control.

Transformation of cotyledon explants of tomato with *Agrobacterium tumefaciens* GC2260 harbouring the vector pBINT1tot or pBINT1m or pBINGUS and the molecular identification of transformed plant will be the objective of future experiments.

# DISCUSSION

A successful nutrition intervention could have a profound impact on the improvement in the quality of life. Evidence for 'food' prevention is derived from animal testing and *in vitro* assays, whereas human clinical trials are still scarce. Some key issues such as bioavailability, metabolism, dose/response and toxicity of these food bioactive compounds have to be well established and a great deal of research is needed to determine the mechanisms by which natural compounds present in human diet may delay or prevent the onset of diseases.

In the present research, we investigated the biological action of two tomato cystine-knot miniproteins which are present in the edible parts of tomato and consequently are components of our diet. Our attention was addressed to angiogenesis, since the identification of new angiogenesis inhibitors endowed with low toxicity may represent a relevant goal in cancer prevention and therapy.

Our characterization of the cystine-rich miniproteins derived from tomato (TCMP-1 and TCMP-2) first considered the expression pattern of the two proteins. Until now, few studies have been performed to investigate cystine-knot miniprotein expression in tomato during fruit development. Previous investigations reported that TCMP-1 is present at high level in the tomato ovary (Martineau *et al*., 1991) and the mRNA of a putative sulfur-rich protein accumulates in tomato fruit (Pear *et al*., 1989). Our results show that TCMP-1 gene is not constantly expressed in all flower development stages, but has an increasing trend during flower bud development, with the highest mRNA level in flower buds 1-3 days before anthesis. After anthesis, the expression of TCMP-1 gene is down-regulated. The expression pattern of TCMP-1 protein is shifted with respect to gene expression, since the highest level of protein was detected in flower buds 4-5 days after anthesis. TCMP-2 gene is expressed at the onset of fruit development and reaches the maximum level in tomato mature fruit. TCMP-2 protein is detectable only in the fruit. Our data indicate that TCMP-1 protein plays its role during tomato flower development and in the first phases of fruit growth, whereas TCMP-2 is a fruit specific protein that accumulates in the fruit at the stage of maturation.

The biological characterization of tomato cystine-knot miniproteins was carried out using recombinant TCMP-1 and TCMP-2 expressed in a bacterial system as well as proteins

extracted from tomato fruits. The recombinant expression of TCMP-1 and TCMP-2 represented an important challenge due to the small size of the proteins and the presence of disulfide bonds, which may affect protein solubility inside the bacterial cell and lead to misfolding and subsequent aggregation. We tested different approaches for the successful recombinant expression and the better strategy results for both miniproteins in the use of the specialized expression vector pET12b based on the T7 promoter-driven system originally developed by Studier and colleagues (Studier *et al*., 1990). Two different host strains were used for optimizing the expression of TCMP-1 and TCMP-2. High yield of TCMP-1 was achieved in OrigamiB (DR3), a bacterial strain which carries glutathione reductase and thioredoxin reductase mutations that enhance the formation of disulfide bonds in the *E. coli* cytoplasm. For TCMP-2 the highest expression was obtained in BL21(DR3) pLysS, a strain that contains a compatible plasmid that provides a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase for a high-stringency expression. Both TCMP-1 and TCMP-2 are accumulated in inclusion bodies. After denaturation of the inclusion bodies, the renaturation in a refolding buffer containing redox agents was proved to be good choice, as it was previously described for the refolding of recombinant potato carboxypeptidase inhibitor (Bronsoms *et al*., 2003). Isolation of TCMPs from mature tomato fruits is favoured by the natural stability and resistance to proteolytic attack of the two miniproteins. However, the purification process is quite long and laborious and requires the processing of a large amount of tomato fruits. The yield of production is about 6.6 mg/kg tomato fruit.

To explore the effects of TCMPs on endothelial cells, we first analyzed the effects of these cystine-knot miniproteins on *in vitro* angiogenesis using both human umbilical vein endothelial cells (HUVEC) and human progenitor endothelial cells. We have seen that TCMPs extracted from tomato and recombinant TCMP-1 and TCMP-2 exert the same biological action, that is they are able to strongly inhibit tube formation. Both recombinant and fruit-extracted TCMPs work at nanomolar concentration, suggesting biological relevance. Neither the N-terminal nor the C-terminal part of TCMP-1 possess biological activity *per se*, so we can assume that for the anti-angiogenic activity, the integrity of the mature part of the miniproteins is required. The presence of intra-molecular disulfide bonds might be important for the biological function of TCMPs.

The emergence and maturation of new vessels are extremely complex and coordinated processes requiring successive activation of cellular events coordinated by numerous signals. Angiogenesis begins from local destruction of the wall of preexisting blood vessel, activation of endothelial cell proliferation, and migration. Every single event is a possible target for an anti-angiogenic effect and to clarify the effect of TCMPs we first investigated the effects of TCMPs on cell proliferation and viability. The present research demonstrates that inhibition of angiogenesis by TCMPs is not due to an interference in cell physiological state. TCMPs inhibit angiogenesis in *in vitro* 2D tube formation assay without altering HUVEC proliferative capability and without increasing cell apoptosis. Moreover, TCMPs are not toxic for endothelial cells, because the viability of HUVEC cells, even in the presence of TCMPs concentrations higher than those necessary for inhibiting angiogenesis, is not altered.

It is well known that the angiogenic process requires, like other biological processes, successive activation of rather a large series of receptors and numerous ligands and a finely adjusted balance between multiple stimulating and inhibitory signals. One of the most important signal pathways is mediated by VEGF receptor and its ligand VEGF, but many other signaling pathways beyond this are implicated in the formation of new vessels and angiogenic response. They include fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF) and several anti-angiogenic factors. The role of EGFR signaling pathway in angiogenesis has been the object of several studies over the past few years. These studies have shown both a direct and an indirect role of EGF-mediated signaling in angiogenesis (Mehta and Besner, 2007; Fujiyama *et al.*, 2001). Blanco-Aparicio *et al*. reported that potato carboxypeptidase inhibitor (PCI), a cystine-knot miniprotein, is an antagonist of EGF. It competed with EGF for binding to EGFR and inhibited EGFR activation and cell proliferation induced by this growth factor (Blanco-Aparicio *et al*., 1998). In a subsequent study it was shown that PCI at micromolar concentration interferes with EGFR activation through the inhibition of receptor dimerization, receptor internalization, and receptor transphosphorylation induced by EGF (Sitjà-Arnau *et al.*, 2005). TCMP-1 is highly homologous the cystine-knot miniproteins PCI of potato (PCI) and TCMP-2 shows a high similarity with members of the cystine-knot miniprotein family identified in potato tubers. Based on these similarities, the hypothesis that the anti-angiogenic action displayed by TCMPs could be linked to inhibition of EGFR phosphorylation is reasonable. However, this hypothesis was not supported by our experimental data. Nanomolar concentrations of TCMPs did not depress EGFR phosphorylation induced by EGF

in A431 cell lines and did not depress phosphorylation of the downstream protein Akt. However, in both HUVEC and A431 lines threonine phosphorylation of ERK1/2 was inhibited. Ras/MAPK and PI3K/Akt pathways are the transduction pathways primarily involved downstream EGFR activation, and phosphorylation of ERK1/2 occurs after the engagement of EGFR in human endothelial cells (Mehta *et al.*, 2007). However, phosphorylation of ERK1/2 occurs also after receptor activation by other ligands, such as fibroblast growth factor (FGF) or VEGF (Slevin *et al.*, 2000). Our data cannot exclude a direct or indirect action of TCMPs on MAP kinase signaling triggered by either FGF or VEGF.

The alternative hypothesis of the TCMPs' involvement in angiogenesis inhibition is supported by their biochemical activity as metallo carboxypeptidase inhibitors. TCMP-1 and PCI are known to be inhibitors of metallo carboxypeptidases and we have shown that also TCMP-2 possesses such biochemical activity. The formation of new vessels requires degradation of cytoskeleton extracellular matrix and the removal of obstructing matrix proteins to allow the formation of the vessel lumen. The proteolytic activity of matrix metalloproteinases (MMPs) are a key component of these processes, both in physiological and pathological conditions (Visse and Nagase, 2003; Galis and Khatri, 2002). The biological activity of MMPs must be tightly regulated if we consider that the MMP family is capable of degrading all the individual component of blood vessel extracellular matrix. Recent efforts have focused on finding ways to control the action of vascular MMPs through the use of nonspecific synthetic inhibitors (Galis *et al*., 1998; Zempo *et al*., 1994) or of natural inhibitors (Allaire *et al*., 1998; Baker *et al*., 1998; Forough *et al*., 1998). It will be interesting to test whether the metalloendopeptidase inhibitor activity of TCMP proteins contributes to their anti-angiogenic properties.

To summarise, we have demonstrated that tomato cystine-knot carboxypeptidase inhibitors both native and recombinant, possess anti-angiogenic activity *in vitro*. The tomato miniproteins act as an anti-angiogenic factor in the nanomolar concentration range, which is of particular biological relevance. Since tomato cystine-knot carboxypeptidase inhibitors are normal components of edible fruits, these compounds should be endowed with low toxicity when tested in animal models of disease and in humans. Tumorigenic angiogenesis could represent a target for therapeutic intervention with tomato miniproteins, and their anti-angiogenic activity could be tested as well for the prevention of other diseases. On these

bases, miniproteins from tomato may be of potential pharmacological interest and deserve further investigation.

# REFERENCES


Aicher TD Buszek KR, Fang FG, Forsyth CJ, Jung SH, Kishi Y, Matelich MC, Scola PM, Spero DM, Yoon SK. (**1992**) Total synthesis of halichondrin B and norhalichondrin B. *J Am Chem Soc*. 114(8):3162-4

Allaire E, Forough R, Clowes M, Starcher B, Clowes AW. (**1998**) Local overexpression of TIMP-1 prevents aortic aneurysm degeneration and rupture in a rat model. *J Clin Invest*. 102(7):1413-20

Allgayer H, Babic R, Gruetzner KU, Tarabichi A, Schildberg FW, Heiss MM. (**2000**) c-erbB-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor-associated protease systems. *J Clin Oncol*. 18(11):2201-9

Al-Nedawi K, Meehan B, Kerbel RS, Allison AC, Rak J. (**2009**) Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc Natl Acad Sci U S A*. 106(10):3794-9

Alshatwi AA, Al Obaaid MA, Al Sedairy SA, Al-Assaf AH, Zhang JJ, Lei KY. (**2010**) Tomato powder is more protective than lycopene supplement against lipid peroxidation in rats. *Nutr Res*. 30(1):66-73

Amin AR, Kucuk O, Khuri FR, Shin DM. (**2009**) Perspectives for cancer prevention with natural compounds. *J Clin Oncol*. 27(16):2712-25

Amin DN, Hida K, Bielenberg DR, Klagsbrun M. (**2006**) Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. *Cancer Res*. 66:2173-80

Anantharaman V, Aravind L, Koonin EV. (**2003**) Emergence of diverse biochemical activities in evolutionarily conserved structural scaffolds of proteins. *Curr Opin Chem Biol.* 7(1):12-20

Andl CD, Mizushima T, Oyama K, Bowser M, Nakagawa H, Rustgi AK. (**2004**) EGFR-induced cell migration is mediated predominantly by the JAK-STAT pathway in primary esophageal keratinocytes. *Am J Physiol Gastrointest Liver Physiol*. 287(6):G 1227-37

Avilés FX, Vendrell J, Guasch A, Coll M, Huber R. (**1993**) Advances in metallo-procarboxypeptidases. Emerging details on the inhibition mechanism and on the activation process. *Eur J Biochem*. 211(3):381-9

Baffert F, Le T, Sennino B, Thurston G, Kuo CJ, Hu-Lowe D, McDonald DM. (**2006**) Cellular changes in normal blood capillaries undergoing regression after inhibition of VEGF signaling. *Am J Physiol Heart Circ Physiol*. 290(2):H547-H59

Baker AH, Zaltsman AB, George SJ, Newby AC. (**1998**) Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro: TIMP-3 promotes apoptosis. *J Clin Invest*. 101(6):1478-87

Baselga J, Mendelsohn J. (**1994**) Receptor blockade with monoclonal antibodies as anti-cancer therapy. *Pharmacol Ther*. 64(1):127-54

Batzer AG, Rotin D, Ureña JM, Skolnik EY, Schlessinger J. (**1994)** Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol Cell Biol*. 14(8):5192-201

Bayés A, de la Vega MR, Vendrell J, Aviles FX, Jongsma MA, Beekwilder J. (**2006**) Response of the digestive system of Helicoverpa zea to ingestion of potato carboxypeptidase inhibitor and characterization of an uninhibited carboxypeptidase B. *Insect Biochem Mol Biol*. 36(8):654-64

Bednarek SY, Wilkins TA, Dombrowski JE, Raikhel NV. (**1990**) A carboxyl-terminal propeptide is necessary for proper sorting of barley lectin to vacuoles of tobacco. *Plant Cell*. 2(12):1145-55

Bianco R, Gelardi T, Damiano V, Ciardiello F, Tortora G. (**2007**) Rational bases for the development of EGFR inhibitors for cancer treatment. *Int J Biochem Cell Biol*. 39(7-8):1416-31

Blanco-Aparicio C, Molina MA, Fernandez-Salas E, Frazier ML, Mas JM, Querol E, Aviles FX, de Llorens R. (**1998**) Potato carboxypeptidase inhibitor, a T-knot protein, is an epidermal growth factor antagonist that inhibits tumor cell growth. *J Biol Chem.* 273(20):12370-7

Blum Y, Belting HG, Ellertsdottir E, Herwig L, Lüders F, Affolter M. (**2008**) Complex cell rearrangements during intersegmental vessel sprouting and vessel fusion in the zebrafish embryo. *Dev Biol.* 316(2):312-22

Brabender J, Danenberg KD, Metzger R, Schneider PM, Park J, Salonga D, Hölscher AH, Danenberg PV. (**2001**) Epidermal growth factor receptor and HER2-neu mRNA expression in non-small cell lung cancer Is correlated with survival. *Clin Cancer Res*. 7(7):1850-5

Breinbauer R, Köhn M. (**2003**) Azide-alkyne coupling: a powerful reaction for bioconjugate chemistry. *Chembiochem*. 4(11):1147-9

Bronsoms S, Villanueva J, Canals F, Querol E, Aviles FX. (**2003**) Analysis of the effect of potato carboxypeptidase inhibitor pro-sequence on the folding of the mature protein. *Eur J Biochem*. 270(17):3641-50

Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, Abbruzzese J, Traxler P, Buchdunger E, Radinsky R, Fidler IJ. (**2000**) Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res*. 60(11):2926-35

Burri PH, Tarek MR. (**1990**) A novel mechanism of capillary growth in the rat pulmonary microcirculation. *Anat Rec*. 228(1):35-45

Butler MS. (**2005**) Natural products to drugs: natural product derived compounds in clinical trials. *Nat Prod Rep*. 22(2):162-95 Erratum in: *Nat Prod Rep*. 2006, 23(1):131

Canene-Adams K, Jeffery EH, Clinton SK, Erdman JW. (**2007**). Mechanisms behind anti-tumor activity in dunning R3327-H prostate adenocarcinomas as a result of tomato & broccoli consumption. *Faseb J.* 21(5):A59

Cardoso LS, Araujo MI, Goes AM, Pacifico LG, Oliveira RR, Oliveira SC. (**2007**) Polymyxin B as inhibitor of LPS contamination of Schistosoma mansoni recombinant proteins in human cytokine analysis. *Microb Cell Fac.* 6:1

Carmeliet P. (**2005**) Angiogenesis in life, disease and medicine. *Nature.* 438(7070):932-6

Carpenter G, Cohen S. (**1990**) Epidermal growth factor. *J Biol Chem.* 265(14):7709-12

Cavallini C. (**2005**) Espressione e purificazione di due inibitori di metallo carbossipeptidasi di pomodoro, TMCP-1 e TMCP-2. Tesi di laurea.

Cerniglia GJ, Pore N, Tsai JH, Schultz S, Mick R, Choe R, Xing X, Durduran T, Yodh AG, Evans SM, Koch CJ, Hahn SM, Quon H, Sehgal CM, Lee WM, Maity A. (**2009**) Epidermal growth factor receptor inhibition modulates the microenvironment by vascular normalization to improve chemotherapy and radiotherapy efficacy. *PLoS One*. 4(8):e6539

Chan S. (**2004**) Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer. *Br J Cancer*. 91(8):1420-4

Charest MG, Lerner CD, Brubaker JD, Siegel DR, Myers AG. (**2005**) A convergent enantioselective route to structurally diverse 6-deoxytetracycline antibiotics. *Science*. 308(5720):395-8

Chiche L, Heitz A, Gelly JC, Gracy J, Chau PT, Ha PT, Hernandez JF, Le-Nguyen D. (**2004**) Squash inhibitors: from structural motifs to macrocyclic knottins. *Curr Protein Pept Sci.* 5(5):341-9

Christmann A, Walter K, Wentzel A, Krätzner R, Kolmar H. (**1999**) The cystine knot of a squash-type protease inhibitor as a structural scaffold for Escherichia coli cell surface display of conformationally constrained peptides. *Protein Eng.* 12(9):797-806

Chua RA, Arbiser JL. (**2009**) The role of angiogenesis in the pathogenesis of psoriasis. *Autoimmunity*. 42(7):574-9

Ciardiello F, Bianco R, Damiano V, Fontanini G, Caputo R, Pomatico G, De Placido S, Bianco AR, Mendelsohn J, Tortora G. (**2000**) Antiangiogenic and antitumor activity of anti-epidermal growth factor receptor C225 monoclonal antibody in combination with vascular endothelial growth factor antisense oligonucleotide in human GEO colon cancer cells. *Clin Cancer Res*. 6(9):3739-47

Ciardiello F, Caputo R, Bianco R, Damiano V, Fontanini G, Cuccato S, De Placido S, Bianco AR, Tortora G. (**2001**) Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res*. 7(5):1459-65

Claxton S, Fruttiger M. (**2004**) Periodic Delta-like 4 expression in developing retinal arteries. *Gene Expr Patterns*. 5(1):123-7

Clore GM, Gronenborn AM, Nilges M, Ryan CA. (**1987**) Three-dimensional structure of potato carboxypeptidase inhibitor in solution. A study using nuclear magnetic resonance, distance geometry, and restrained molecular dynamics. *Biochemistry*. 26(24):8012-23

Cohen LA. (**2002**) Nutrition and prostate cancer: a review. *Ann N Y Acad Sci*. 963:148-55

Colgrave ML, Craik DJ. (**2004)** Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot. *Biochemistry*. 43(20):5965-75

Craik DJ, Cemazar M, Daly NL. (**2007**) The chemistry and biology of cyclotides. *Curr Opin Drug Dicov Devel*. 10(2):176-84

Craik DJ, Cemazar M, Wang CK, Daly NL. (**2006**) The cyclotide family of circular miniproteins: nature's combinatorial peptide template. *Biopolymers*. 84(3):250-66

Craik DJ, Daly NL, Bond T, Waine C. (**1999**) Plant cyclotides: A unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. *J Mol Biol*. 294(5):1327-36

Craik DJ, Simonsen S, Daly NL. (**2002**) The cyclotides: novel macrocyclic peptides as scaffolds in drug design. *Curr Opin Drug Discov Devel*. 5(2):251-60

Craik DJ. (**2001**). Plant cyclotides: circular, knotted peptide toxins. *Toxicon*. 39(12):1809-13.

Crawford TN, Alfaro DV 3rd, Kerrison JB, Jablon EP. (**2009**) Diabetic retinopathy and angiogenesis. *Curr Diabetes Rev*. 5(1):8-13

Cwirla SE, Balasubramanian P, Duffin DJ, Wagstrom CR, Gates CM, Singer SC, Davis AM, Tansik RL, Mattheakis LC, Boytos CM, Schatz PJ, Baccanari DP, Wrighton NC, Barrett RW, Dower WJ. (**1997**) Peptide agonist of the thrombopoietin receptor as potent as the natural cytokine. *Science*. 276(5319):1696-9

Daher IN, Yeh ET. (**2008**) Vascular complications of selected cancer therapies. *Nat Clin Pract Cardiovasc Med*. 5(12):797-805

Dälken B, Giesübel U, Knauer SK, Wels WS. (**2006**) Targeted induction of apoptosis by chimeric granzyme B fusion proteins carrying antibody and growth factor domains for cell recognition. *Cell Death Differ*. 13(4):576-85

Daopin S, Piez KA, Ogawa Y, Davies DR. (**1992**) Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. *Science*. 257(5068):369-73

Dejana E, Tournier-Lasserve E, Weinstein BM. (**2009**) The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell*. 16(2):209-21

Díez-Díaz M, Conejero V, Rodrigo I, Pearce G, Ryan CA. (**2004**) Isolation and characterization of wound-inducible carboxypeptidase inhibitor from tomato leaves. *Phytochemistry*. 65(13):1919-24

Djonov VG, Kurz H, Burri PH. (**2002**) Optimality in the developing vascular system: branching remodeling by means of intussusception as an efficient adaptation mechanism. *Dev Dyn*. 224(4):391-402

Dutta PR, Maity A. (**2007**) Cellular responses to EGFR inhibitors and their relevance to cancer therapy. *Cencer Lett*. 254(2):165-77

Elice F, Rodeghiero F, Falanga A, Rickles FR. (**2009**) Thrombosis associated with angiogenesis inhibitors. *Best Pract Res Clin Haematol*. 22(1):115-28

Ellis LM. (**2004**) Epidermal growth factor receptor in tumor angiogenesis. *Hematol Oncol Clin North Am*. 18(5):1007-21, viii

Ennis BW, Lippman ME, Dickson RB. (**1991**) The EGF receptor system as a target for antitumor therapy. *Cancer Invest*. 9(5):553-62

Epstein RJ. (**2007**) VEGF signaling inhibitors: more pro-apoptotic than anti-angiogenic. *Cancer Metastasis Rev*. 26(3-4):443-52

Espín JC, García-Conesa MT, Tomás-Barberán FA. (**2007**) Nutraceuticals: facts and fiction. *Phytochemistry*. 68(22-24):2986–3008

Etminan M, Takkouche B, Caamaño-Isorna F. (**2004**). The role of tomato products and lycopene in the prevention of prostate cancer: A meta-analysis of observational studies. *Cancer Epidemiol Biomarkers Prev.* 13(3):340-5

Ferrara N, Gerber HP, LeCouter J. (**2003**) The biology of VEGF and its receptors. *Nat Med*. 9:669-76

Ferrara N, Gerber HP. (**2001**) The role of vascular endothelial growth factor in angiogenesis. *Acta Haematol.* 106(4):148-56

Ferrara N, Hillan KJ, Gerber HP, Novotny W. (**2004**) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov*. 3(5):391-400

Ferrara N. (**2002**) VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer*. 2(10):795-803

Folkman J. (**1971**) Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 285(21):1182-6

Folkman J. (**1995**) Angiogenesis in Cancer, Vascular, Rheumatoid and Other Disease. *Nat Med.* 1(1):27-31.

Folkman J. (**2006**) Angiogenesis. *Annu Rev Med*. 57:1-18

Forough R, Lea H, Starcher B, Allaire E, Clowes M, Hasenstab D, Clowes AW. (**1998**) Metalloproteinase blockade by local overexpression of TIMP-1 increases elastin accumulation in rat carotid artery intima. *Arterioscler Thromb Vasc Biol*. 18(5):803-7

Fraisl P, Mazzone M, Schmidt T, Carmeliet P. (**2009**) Regulation of angiogenesis by oxygen and metabolism. *Dev Cell*. 16(2):167-79

Freeman M, Klämbt C, Goodman CS, Rubin GM. (**1992**) The argos gene encodes a diffusible factor that regulates cell fate decision in the Drosophila eye. *Cell*. 69(6):963-75

Fruttiger M. (**2007**) Development of the retinal vasculature. *Angiogenesis*. 10(2):77-88

Fujiyama S, Matsubara H, Nozawa Y, Maruyama K, Mori Y, Tsutsumi Y, Masaki H, Uchiyama Y, Koyama Y, Nose A, Iba O, Tateishi E, Ogata N, Jyo N, Higashiyama S, Iwasaka T. (**2001**) Angiotensin AT(1) and AT(2) receptors differentially regulate angiopoietin-2 and vascular endothelial growth factor expression and angiogenesis by modulating heparin binding-epidermal growth factor (EGF)-mediated EGF receptor transactivation. *Circ Res.* 88(1):22-9

Galis ZS, Asanuma K, Godin D, Meng X. (**1998**) *N*-acetyl-cysteine decreases the matrix-degrading capacity of macrophage-derived foam cells: new target for antioxidant therapy?. *Circulation*. 97(24):2445-53

Galis ZS, Khatri JJ. (**2002**) Matrix metalloproteinases in vascular remodelling and atherogenesis: the good, the bad, and the ugly. *Circ Res*. 90(3):251-62

Gavin AC, Nebreda AR. (**1999**) A MAP kinase docking site is required for phosphorylation and activation of p90(rsk)/MAPKAP kinase-1. *Curr Biol*. 9(5):281-4

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C. (**2003**) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol*. 161(6):1163-77

Gillaspy G, Ben-David H, Gruissem W. (**1993**) Fruits: a developmental perspective. *Plant Cell*. 5(10):1439-51

Glade MJ. (**1999**) Food, nutrition, and the prevention of cancer: A global perspective: American Institute for Cancer Res/World Cancer Res Fund, American Institute for Cancer Res, 1997. *Nutrition*. 15(6):523-6

Goldman CK, Kim J, Wong WL, King V, Brock T, Gillespie GY. (**1993**) Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol Biol Cell*. 4(1):121-33

Göransson U, Sjögren M, Svangård E, Claeson P, Bohlin L. (**2004**) Reversible antifouling effect of the cyclotide cycloviolacin O2 against barnacles. *J Nat Prod*. 67(8):1287-90

Gordaliza M. (**2007**) Natural products as leads to anticancer drugs. *Clin Transl Oncol*. 9(12):767-76

Groenen LC, Nice EC, Burgess AW. (**1994**) Structure-function relationships for the EGF/TGF-alpha family of mitogens. *Growth Factors*. 11(4):235-57

Gustafson KR, McKee TC, Bokesch HR. (**2004**) Anti-HIV cyclotides. *Curr Protein Pept Sci.* 5(5):331-40

Hanahan D, Folkman J. (**1996**) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 86(3):353-64

Hanahan D, Weinberg RA. (**2000**) The hallmarks of cancer. *Cell*. 100(1):57-70

Hass GM, Derr JE, Makus DJ, Ryan CA. (**1979a**) Purification and characterization of the carboxypeptidase isoinhibitors from potatoes. *Plant Physiol*. 64(6):1022-28

Hass GM, Derr JE, Makus DJ. (**1979b**) Distribution of carboxypeptidase isoinhibitors in potato plant. *Plant Physiol*. 64(6):1029-31

Hass GM, Hermodson MA. (**1981**) Amino acid sequence of a carboxypeptidase inhibitor from tomato fruit. *Biochemistry*. 20(8):2256-60

Hass GM, Nau H, Biemann K, Grahn DT, Ericsson LH, Neurath H. (**1975**) The amino acid sequence of a carboxypeptidase inhibitor from potatoes. *Biochemistry*. 14:1334-42

Hass GM, Ryan CA. (**1980**) carboxypeptidase inhibitor from ripened tomatoes purification and properties. *Phytochemistry*. 19:1329-33

Hearn MT, Gomme PT. (**2000**) Molecular architecture and biorecognition processes of the cystine knot protein superfamily: part I. The glycoprotein hormones. *J Mol Recognit*. 13(5):223-78

Heitz A, Avrutina O, Le-Nguyen D, Diederichsen U, Hernandez JF, Gracy J, Kolmar H, Chiche L. (**2008**) Knottin cyclization: impact on structure and dynamics. *BMC Struct Biol*. 8:54

Hicklin DJ, Ellis LM. (**2005**) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol*. 23(5):1011-27

Hirata A, Ogawa S, Kometani T, Kuwano T, Naito S, Kuwano M, Ono M. (**2002**) ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res*. 62(9):2554-60

Holländer-Czytko H, Andersen JK, Ryan CA. (**1985**) Vacuolar Localization of Wound-Induced Carboxypeptidase Inhibitor in Potato Leaves. *Plant Physiol*. 78(1):76-9

Holtz DO, Krafty RT, Mohamed-Hadley A, Zhang L, Alagkiozidis I, Leiby B, Guo W, Gimotty PA, Coukos G. (**2008**) Should tumor VEGF expression influence decisions on combining low-dose chemotherapy with antiangiogenic therapy? Preclinical modeling in ovarian cancer. *J Transl Med*. 6:2

Huang SM, Li J, Armstrong EA, Harari PM. (**2002**) Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa). *Cancer Res*. 62(15):4300-6

Hughes S, Chang-Ling T. (**2000**) Roles of endothelial cell migration and apoptosis in vascular remodeling during development of the central nervous system. *Microcirculation*. 7(5):317-33

Hwang ES, Bowen PE. (**2002**) Can the consumption of tomatoes or lycopene reduce cancer risk? *Integr Cancer Ther*. 1(2):121-32; discussion 132

Ireland DC, Colgrave ML, Craik DJ. (**2006**) A novel suite of cyclotides from Viola odorata: sequence variation and the implications for structure, function and stability. *Biochem J.* 400(1):1-12

Iruela-Arispe ML, Davis GE. (**2009**) Cellular and molecular mechanisms of vascular lumen formation. *Dev Cell*. 16(2):222-31

Jain RK. (**2003**) Molecular regulation of vessel maturation. *Nat Med*. 9(6):685-93

Jennings C, West J, Waine C, Craik D, Anderson M. (**2001**) Biosynthesis and insecticidal properties of plant cyclotides: the cyclic knotted proteins from Oldenlandia affinis. Proc Natl Acad Sci U S A. 98(19):10614-9

Johnson GL, Dohlman HG, Graves LM. (**2005**) MAPK kinase kinases (MKKKs) as a target class for small-molecule inhibition to modulate signaling networks and gene expression. *Curr Opin Chem Biol*. 9(3):325-31

Jones EA, le Noble F, Eichmann A. (**2006**) What determines blood vessel structure? Genetic prespecification vs. hemodynamics. *Physiology (Bethesda)*. 21:388-95

Kane LP, Shapiro VS, Stokoe D, Weiss A. (**1999**) Induction of NF-kappaB by the Akt/PKB kinase. *Curr Biol*. 9(11):601-4

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. (**1993**) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature*. 362(6423):841-4

Kimura RH, Cheng Z, Gambhir SS, Cochran JR. (**2009**) Engineered knottin peptides: a new class of agents for imaging integrin expression in living subjects. *Cancer Res*. 69(6):2435-42

Klagsbrun M, D'Amore PA. (**1991**) Regulators of angiogenesis. *Annu Rev Physiol*. 53:217-39

Klein DE, Stayrook SE, Shi F, Narayan K, Lemmon MA. (**2008**) Stuctural basis for EGFR ligand sequestration by Argos. *Nature*. 453(7199):1271-5

Kloth MT, Laughlin KK, Biscardi JS, Boerner JL, Parsons SJ, Silva CM. (**2003**) STAT5b, a Mediator of Synergism between c-Src and the Epidermal Growth Factor Receptor. *J Biol Chem*. 278(3):1671-9

Krätzner R, Debreczeni JE, Pape T, Schneider TR, Wentzel A, Kolmar H, Sheldrick GM, Uson I. (**2005**) Structure of Ecballium elaterium trypsin inhibitor II (EETI-II): a rigid molecular scaffold. *Acta Crystallogr D Biol Crystallogr*. 61(Pt 9):1255-62

Krause S, Schmoldt HU, Wentzel A, Ballmaier M, Friedrich K, Kolmar H. (**2007**) Grafting of thrombopoietin-mimetic peptides into cystine knot miniproteins yields high-affinity thrombopoietin antagonists and agonists. *Febs J.* 274(1):86-95

Kulik G, Klippel A, Weber MJ. (**1997**) Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Mol Cell Biol*. 17(3):1595-606

Kurz H. (**2000**) Physiology of angiogenesis. *J Neurooncol*. 50(1-2):17-35

Le Nguyen D, Heitz A, Chiche L, Castro B, Boigegrain RA, Favel A, Coletti-Previero MA. (**1990**) Molecular recognition between serine proteases and new bioactive microproteins with a knotted structure. *Biochimie.* 72(6-7):431-5

Leaman DW, Pisharody S, Flickinger TW, Commane MA, Schlessinger J, Kerr IM, Levy DE, Stark GR. (**1996**) Roles of JAKs in activation of STATs and stimulation of c-fos gene expression by epidermal growth factor. *Mol Cell Biol*. 16(1):369-75

Lee M, Aoki M, Kondo T, Kobayashi K, Okumura K, Komori K, Murohara T. (**2005**) Therapeutic angiogenesis with intramuscular injection of low-dose recombinant granulocyte-colony stimulating factor. *Arterioscler Thromb Vasc Biol*. 25(12):2535-41

Lerner DR, Raikhel NV. (**1989**) Cloning and characterization of root-specific barley lectin. *Plant Physiol*. 91(1):124-129

Leslie JD, Ariza-McNaughton L, Bermange AL, McAdow R, Johnson SL, Lewis J. (**2007**) Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. *Development*. 134(5):839-44

Levitzki A. (**1996**) Targeting signal transduction for disease therapy. *Curr Opin Cell Biol*. 8(2):239-44

Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG. (**2004**) A small molecule Smac mimic potentiates TRAIL-and TNF alpha-mediated cell death. *Science*. 305(5689):1471-4

Liang WC, Wu X, Peale FV, Lee CV, Meng YG, Gutierrez J, Fu L, Malik AK, Gerber HP, Ferrara N, Fuh G. (**2006**) Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. *J Biol Chem*. 281(2):951-61

Lila MA. (**2007**) From beans to berries and beyond: teamwork between plant chemicals for protection of optimal human health. *Ann N Y Acad Sci*. 1114:372-80

Lin H, Chen C, Li X, Chen BD. (**2002**) Activation of the MEK/MAPK pathway is involved in bryostatin1-induced monocytic differenciation and up-regulation of X-linked inhibitor of apoptosis protein. *Exp Cell Res*. 272(2):192-8

Lindholm P, Gullbo J, Claeson P, Göransson U, Johansson S, Backlund A, Larsson R, Bohlin L. (**2002**) Selective cytotoxicity evaluation in anticancer drug screening of fractionated plant extracts. *J Biomol Screen*. 7(4):333-40

Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar-Sagi D, Schlessinger J. (**1992**) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell*. 70(3):431-42

Lucitti JL, Jones EA, Huang C, Chen J, Fraser SE, Dickinson ME. (**2007**) Vascular remodeling of the mouse yolk sac requires hemodynamic force. *Development*. 134(18):3317-26

Mahady GB. (**2001**) Global harmonization of herbal health claims. *J Nutr*. 131(3s):1120S-3S

Maity A, Pore N, Lee J, Solomon D, O'Rourke DM. (**2000**) Epidermal growth factor receptor transcriptionally up-regulates vascular endothelial growth factor expression in human glioblastoma cells via a pathway involving phosphatidylinositol 3'-kinase and distinct from that induced by hypoxia. *Cancer Res*. 60(20):5879-86

Manson MM, Farmer PB, Gescher A, Steward WP. (**2005**) Innovative agents in cancer prevention. *Recent Results Cancer Res.* 166:257-75

Marais R, Marshall CJ. (**1996**) Control of the ERK MAP kinase cascade by Ras and Raf. *Cancer Surv*. 27:101-25

Martineau B, McBride KE, Houck CM. (**1991**) Regulation of metallocarboxypeptidase inhibitor gene expression in tomato. *Mol Gen Genet.* 228(1-2):281-6

McDonald NQ, Lapatto R, Murray-Rust J, Gunning J, Wlodawer A, Blundell TL. (**1991**) New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. *Nature*. 354(6352):411-4

Mehta VB, Besner GE. (**2007**) HB-EGF promotes angiogenesis in endothelial cells via PI3-kinase and MAPK signaling pathways. *Growth Factors.* 25(4):253-63

Mendelsohn J, Dinney CP. (**2001**) The Willet F. Whitmore, Jr., Lectureship: blockade of epidermal growth factor receptors as anticancer therapy. *J Urol*. 165(4):1152-7

Michaelis UR, Fisslthaler B, Medhora M, Harder D, Fleming I, Busse R. (**2003**) Cytochrome P450 2C9-derived epoxyeicosatrienoic acids induce angiogenesis via cross-talk with the epidermal growth factor receptor (EGFR). *FASEB J.* 17(6):770-2

Miyata T. (**2007**) Pharmacological basis of traditional medicines and health supplements as curatives. *J Pharmacol Sci*. 103(2):127-31

Molina MA, Avilés FX, Querol J. (**1992**) Expression of a synthetic gene encoding potato carboxypeptidase inhibitor using a bacterial secretion vector. *Gene*. 116(2):129-38

Moroni MC, Willingham MC, Beguinot L. (**1992**) EGF-R antisense RNA blocks expression of the epidermal growth factor receptor and suppresses the transforming phenotype of a human carcinoma cell line. *J Biol Chem*. 267(4):2714-22

Murphy LO, Smith S, Chen RH, Fingar DC, Blenis J. (**2002**) Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol*. 4(8):556-64

Muto Y, Suzuki K, Sato E, Ishii H. (**2003**) Carboxypeptidase B inhibitors reduce tissue factor-induced renal microthrombi in rats. *Eur J Pharmacol*. 461(2-3):181-9

Nelson J, Stewart R, McGivern M, Bailie JR, Walker B, Murphy RF, Wilson DJ. (**1991**) Synthetic murine epidermal growth factor sequence 20-31 is mitogenic and angiogenic. *Carcinogenesis*. 12(10):1823-9

Newman DJ, Cragg GM, Snader KM. (**2003**) Natural products as sources of new drugs over the period 1981-2002. *J Nat Prod*. 66(7):1022-37

Nishino H, Satomi Y, Tokuda H, Masuda M. (**2007**) Cancer control by phytochemicals. *Curr Pharm Des*. 13(33):3394-9

Noonberg SB, Benz CC. (**2000**) Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily: role as anticancer agents. *Drugs*. 59(4):753-62

Norton RS, Pallaghy PK. (**1998**) The cystine knot structure of ion channel toxins and related polypeptides. *Toxicon*. 36(11):1573-83

Novak JZ. (**2006**) Age-related macular degeneration (AMD): pathogenesis and therapy. *Pharmacol Rep*. 58(3):353-63

Oefner C, D'Arcy A, Winkler FK, Eggimann B, Hosang M. (**1992**) Crystal structure of human platelet-derived growth factor BB. *EMBO J*. 11(11):3921-6

Pallaghy PK, Nielsen KJ, Craik DJ, Norton RS. (**1994**) A common structural motif incorporating a cystine knot and a triple-stranded beta-sheet in toxic and inhibitory polypeptides. *Protein Sci*. 3(10):1833-9

Parikh AA, Liu WB, Fan F, Stoeltzing O, Reinmuth N, Bruns CJ, Bucana CD, Evans DB, Ellis LM. (**2003**) Expression and regulation of the novel vascular endothelial growth factor receptor neuropilin-1 by epidermal growth factor in human pancreatic carcinoma. *Cancer*. 98(4):720-9

Paterson I, Florence GJ. (**2003**) The development of a practical total synthesis of discodermolide, a promising microtubule-stabilizing anticancer agent. *Eur J Org Chem*. 2003(12):2193-208

Pear JR, Ridge N, Rasmussen R, Rose RE, Houck CM. (**1989**) Isolation and characterisation of a fruit-specific cDNA and the corresponding genomic clone from tomato. *Plant Mol Biol.* 13(6):639-51

Perlman D, Halvorson HO. (**1983**) A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J Mol Biol*. 167(2):391-409

Perrotte P, Matsumoto T, Inoue K, Kuniyasu H, Eve BY, Hicklin DJ, Radinsky R, Dinney CP. (**1999**) Anti epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res*. 5(2):257-65

Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B, Kerbel RS. (**1997**) Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol*. 151(6):1523-30

Pore N, Jiang Z, Gupta A, Cerniglia G, Kao GD, Maity A. (**2006**) EGFR tyrosine kinase inhibitors decrease VEGF expression by both hypoxia-inducible factor (HIF)-1-independent and HIF-1-dependent mechanisms. *Cancer Res*. 66(6):3197-204

Pore N, Liu S, Haas-Kogan DA, O'Rourke DM, Maity A. (**2003**) PTEN mutation and epidermal growth factor receptor activation regulate vascular endothelial growth factor (VEGF) mRNA expression in human glioblastoma cells by transactivating the proximal VEGF promoter. *Cancer Res*. 63(1):236-41

Quilis J, Meynard D, Vila L, Avilés FX, Guiderdoni E, San Segundo B. (**2007**) A potato carboxypeptidase inhibitor gene provides pathogen resistance in transgenic rice. *Plant Biotechnol J.* 5(4):537-53

Raikhel NV, Wilkins TA. (**1987**) Isolation and characterization of a cDNA clone encoding wheat germ agglutinin. *Proc Natl Acad Sci U S A*. 84(19):6745-6749

Rees DC, Lipscomb WN. (**1980**) Structure of potato inhibitor complex of carboxypeptidase A at 5.5-A resolution. *Proc Natl Acad Sci U S A*. 77(1):277-80

Rees DC, Lipscomb WN. (**1982**) Refined crystal structure of the potato inhibitor complex of carboxypeptidase A at 2.5 A resolution. *J Mol Biol*. 160(3):475-98

Riese DJ 2nd, van Raaij TM, Plowman GD, Andrews GC, Stern DF. (**1995**) The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol Cell Biol*. 15(10):5770-6. Erratum in: *Mol Cell Biol.* 1996, 16(2):735

Risau W. (**1997**) Mechanisms of angiogenesis. *Nature*. 386(6626):671-4

Ruck A, Paulie S. (**1998**) EGF, TGF alpha, AR and HB-EGF are autocrine growth factors for human bladder carcinoma cell lines. *Anticancer Res*. 18(3A):1447-52

Salomon DS, Brandt R, Ciardiello F, Normanno N. (**1995**) Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol*. 19(3):183-232

Sapieha P, Hamel D, Shao Z, Rivera JC, Zaniolo K, Joyal JS, Chemtob S. (**2010**) Proliferative retinopathies: angiogenesis that blinds. *Int J Biochem Cell Biol*. 42(1):5-12

Schlunegger MP, Grütter MG. (**1992**) An unusual feature revealed by the crystal structure at 2.2 A resolution of human transforming growth factor-beta 2. *Nature.* 358(6385):430-4

Schöpke T, Agha MI, Wray V, Hiller K. (**1994**) Triterpenoid saponins from Bellium bellidioides. *Phytochemistry*. 36(2):449-53

Shibuya M, Claesson-Welsh L. (**2006**) Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp Cell Res*. 312(5):549-60

Siekmann AF, Lawson ND. (**2007**) Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature.* 445(7129):781-4

Sini P, Wyder L, Schnell C, O'Reilly T, Littlewood A, Brand R, Hynes NE, Wood J. (**2005**) The antitumor and antiangiogenic activiry of vascular endothelial growth factor receptor inhibition is potentiated by ErbB1 blockade. *Clin Cancer Res*. 11:4521-32

Sitjà-Arnau M, Molina MA, Blanco-Aparicio C, Ferrer-Soler L, Lorenzo J, Avilés FX, Querol E, de Llorens R. (**2005**) Mechanism of action of potato carboxypeptidase inhibitor (PCI) as an EGF blocker. *Cancer Lett.* 226(2):169-84

Slevin M, Krupinski J, Slowik A, Rubio F, Szczudlik A, Gaffney J. (**2000**) Activation of MAP kinase (ERK-1/ERK-2), tyrosine kinase and VEGF in the human brain following acute ischaemic stroke. *Neuroreport.* 11(12):2759-64

Smith III AB, Beauchamp TJ, LaMarche MJ, Kaufman MD, Qiu Y, Arimoto H, Jones DR, Kobayashi K. (**2000**) Evolution of a Gram-Scale Synthesis of (+)-Discodermolide. *J Am Chem Soc*. 122(36):8654-64

Song G, Ouyang G, Bao S. (**2005**) The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med*. 9(1):59-71

Stover DR, Becker M, Liebetanz J, Lydon NB. (**1995**) Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and P85 alpha. *J Biol Chem*. 270(26):15591-7

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. (**1990**) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol*. 185:60-89

Sun H, Nikolovska-Coleska Z, Yang CY, Xu L, Liu M, Tomita Y, Pan H, Yoshioka Y, Krajewski K, Roller PP, Wang S. (**2004**) Structure-based design of potent, conformationally constrained Smac mimetics. *J Am Chem Soc*. 126(51):16686-7

Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, Khuri FR. (**2005**) Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res*. 65(16):7052-8

Szekanecz Z, Besenyei T, Paragh G, Koch AE. (**2009**) Angiogenesis in rheumatoid arthritis. *Autoimmunity*. 42(7):563-73

Tammela T, Zarkada G, Wallgard E, Murtomäki A, Suchting S, Wirzenius M, Waltari M, Hellström M, Schomber T, Peltonen R, Freitas C, Duarte A, Isoniemi H, Laakkonen P, Christofori G, Ylä-Herttuala S, Shibuya M, Pytowski B, Eichmann A, Betsholtz C, Alitalo K. (**2008**) Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature*. 454(7204):656-60

Tonucci LH, Holden JM, Beecher GR, Khachik F, Davis CS, Mulokozi G. (**1995**) Carotenoid content of thermally processed tomato-based food products. *J Agric Food Chem*. 43(3):579-86

Trinh XB, Tjalma WA, Vermeulen PB, Van den Eynden G, Van der Auwera I, Van Laere SJ, Helleman J, Berns EM, Dirix LY, van Dam PA. (**2009**) The VEGF pathway and the AKT/mTOR/p70S6K1 signalling pathway in human epithelial ovarian cancer. *Br J Cancer*. 100(6):971-8

USDA Nutrient Data Bank - www.nal.usda.gov

Vainio H, Weiderpass E. (**2006**) Fruit and vegetables in cancer prevention. *Nutr Cancer*. 54(1):111-42

van Cruijsen H, Voest EE, Punt CJ, Hoekman K, Witteveen PO, Meijerink MR, Puchalski TA, Robertson J, Saunders O, Jürgensmeier JM, van Herpen CM, Giaccone G. (**2010**) Phase I evaluation of cediranib, a selective VEGFR signalling inhibitor, in combination with gefitinib in patients with advanced tumours. *Eur J Cancer*. Jan 8 [Epub ahead of print]

Visse R, Nagase H. (**2003**) Matrix metallo proteinases and tissue inhibitors of metallo proteinases: structure, function, and biochemistry. *Circ Res*. 92(8):827-39

Vitt UA, Hsu SY, Hsueh AJ. (**2001**) Evolution and classification of cystine knot-containing hormones and related extracellular signaling molecules. *Mol Endocrinol.* 15(5):681-94

Voice JK, Klemke RL, Le A, Jackson JH. (**1999**) Four human ras homologs differ in their abilities to activate Raf-1, induce transformation, and stimulate cell motility. *J Biol Chem*. 274(24):17164-70

von Heijne G. (**1983**) Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem*. 133(1):17-21

Wang CK, Hu SH, Martin JL, Sjögren T, Hajdu J, Bohlin L, Claeson P, Göransson U, Rosengren KJ, Tang J, Tan NH, Craik DJ. (**2009**) Combined X-ray and NMR analysis of the stability of the cyclotide cystine knot fold that underpins its insecticidal activity and potential use as drug scaffold. *J Biol Chem*. 284(16):10672-83

Wang Q, Chan TR, Hilgraf R, Fokin VV, Sharpless KB, Finn MG. (**2003**) Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J Am Chem Soc*. 125(11):3192-3

Wang X, Smith PL, Hsu MY, Ogletree ML, Schumacher WA. (**2006**) Murine model of ferric chloride-induced vena cava thrombosis: evidence for effect of potato carboxypeptidase inhibitor. *J Thromb Haemost.* 4(2):403-10

Waters SB, Chen D, Kao AW, Okada S, Holt KH, Pessin JE. (**1996**) Insulin and epidermal growth factor receptors regulate distinct pools of Grb2-SOS in the control of Ras activation. *J Biol Chem*. 271(30):18224-30

Wedge SR, Kendrew J, Hennequin LF, Valentine PJ, Barry ST, Brave SR, Smith NR, James NH, Dukes M, Curwen JO, Chester R, Jackson JA, Boffey SJ, Kilburn LL, Barnett S, Richmond GH, Wadsworth PF, Walker M, Bigley AL, Taylor ST, Cooper L, Beck S, Jürgensmeier JM, Ogilvie DJ. (**2005**) AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. *Cancer Res*. 65(10):4389-400

Werle M, Kafedjiiski K, Kolmar H, Bernkop-Schnürch A. (**2007**) Evaluation and improvement of the properties of the novel cystine-knot microprotein McoEeTI for oral administration. *Int J Pharm*. 332(1-2):72-9

Werle M, Kolmar H, Albrecht R, Bernkop-Schnürch A. (**2008**) Characterisation of the barrier caused by luminally secreted gastro-intestinal proteolytic enzymes for two novel cystine-knot microproteins. *Amino Acids*. 35(1):195-200

Werle M, Schmitz T, Huang HL, Wentzel A, Kolmar H, Bernkop-Schnürch A. (**2006**) The potential of cystine-knot microproteins as novel pharmacophoric scaffolds in oral peptide drug delivery. *J Drug Target.* 14(3):137-46

Wilkins TA, Bednarek SY, Raikhel NV. (**1990**) Role of propeptide glycan in post-translational processing and transport of barley lectin to vacuoles in transgenic tobacco. *Plant Cell*. 2(4):301-13

Wilkinson S, Chodak GW. (**2003**) Critical review of complementary therapies for prostate cancer. *J Clin Oncol.* 21(11):2199-210

Williams MT, Hord NG. (**2005**) The role of dietary factors in cancer prevention: beyond fruits and vegetables. *Nutr Clin Pract.* 20(4):451-9

Wright CS. (**1981**) Histidine determination in wheat germ agglutinin isolectin by X-ray diffraction analysis. *J Mol Biol*. 145(2):453-61

Yen L, Benlimame N, Nie ZR, Xiao D, Wang T, Al Moustafa AE, Esumi H, Milanini J, Hynes NE, Pages G, Alaoui-Jamali MA. (**2002**) Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers. *Mol Biol Cell*. 13(11):4029-44

Zempo N, Kenagy RD, Au YPT, Bendeck M, Clowes MM, Reidy MA, Clowes AW. (**1994**) Matrix metalloproteinases of vascular wall cells are increased in balloon-injured rat carotid artery. *J Vasc Surg*. 20(2):209-17

Zhang A, Meng L, Wang Q, Xi L, Chen G, Wang S, Zhou J, Lu Y, Ma D. (**2006**) Enhanced in vitro invasiveness of ovarian cancer cells through up-regulation of VEGF and induction of MMP-2. *Oncol Rep*. 15(4):831-6

Zhang C, DeLisi C. (**1998**) Estimating the number of protein folds. *J Mol Biol*. 284(5):1301-5

Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, Semenza GL. (**2000**) Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res*. 60(6):1541-5

Zumkeller W, Schofield PN. (**1995**) Growth factors, cytokines and soluble forms of receptor molecules in cancer patients. *Anticancer Res*. 15(2):343-8

Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, Giaccia AJ. (**2000**) Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev*. 14(4):391-6

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