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Mini Review

ANTITUMOR POTENTIAL OF PLANT PROTEASE INHIBITORS

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Summary: One of the most important issues facing health care is the prevention and treatment of different types of cancer. Among the most frequently encountered types of cancer leading to lethality are lung cancer, breast cancer and colon cancer. The search for new drugs for cancer cells treatment and clarifying the mechanisms of their anti-tumor effect is a scientific challenge and a necessary basis for new more effective methods of cancer treatment. A major role in tumor growth, invasion, angiogenesis and metastasis have different types of proteases whose activities are inhibited by some synthetic drugs. Protease inhibitors of plant origin have the potential to be an alternative or supplement to the treatment with synthetic drugs, but the mechanisms of their anti-tumor effects are poorly understood. In this review the current knowledge of plant protease inhibitors as tumor preventive and suppresive agents, and the perspectives of their use as antitumour drugs are discussed.

Keywords: Cancer; proteases; protease inhibitors.

Abbreviations: BBPI – Bowman-Birk type protease inhibitor; KTI – Kunitz type protease inhibitor; MMP – matrix metalloprotease; PI – protease inhibitor.

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Content:

1. HUMAN CANCER – STATISTICS, CANCEROGENESIS AND TRADITIONAL METHODS OF TREATMENT

One of the most fundamental healthcare problems is the prevention and the treatment of cancer diseases. Statistically, the cancer is a global leading cause of death – a situation currently worsened by population growth and aging trends, urbanization and strengthening of risk factors. In 2012 alone, 14.1 million

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new cancer disease cases and 8.2 million cases of death due to cancer have been registered (Torre et al., 2015). Lung cancer, breast cancer, prostate cancer and colon cancer are among the most frequently found lethal types. For example, breast cancer is the most frequent cancer type diagnosed among women in Bulgaria, and the second most frequent among women worldwide (http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/breast-cancer-statistics). In all European countries illness rate is high and it has been increasing for the past 5 years, according to the World Health Organization (http://eco.iarc.fr/eucan). During the past decade the frequency of breast cancer in Bulgaria has increased almost twice, and it is now leading malignant tumor type among women (http://www.sbaloncology.bg/bg/bulgarian-cancer-registry.htm, 2015). The peak of this disease is found among women in middle and old age, however the cases of breast cancer among younger individuals under 40-years of age have increased, a fact directly linked to their reproductive health. The prostate cancer is the second cause of death among men (Jemal et al., 2010). The incidence of prostate cancer has the highest rate in the developed countries (Ferlay et al., 2010). The increased breast and prostate cancers frequences in the last years as well as of all types of cancers requires an intense research in direction of effective drugs for cancer prevention and treatment, including exploitation of natural plant-derived compounds (Wang et al., 2012).

The onset of cancer is associated with a number of factors, such as exogenous oxidative stress (Blein et al., 2014), genotoxic stress (D'Assoro et al., 2004), and is influenced both by external environmental factors and internal factors such as age, change in the hormonal status of the individual (Lukong, 2017), heredity, inflammatory processes. Oxidative stress has a role in the process of cancer transformation in all cancer types and the molecular responders of the stress are the same in every type of cancers (Thapa and Ghosh, 2012). The increased free radicals concentration in cells changes cell homeostasis and the end result of these atypical processes could be oncogenic transformation and cancer formation (Federico et al., 2007).

Cancer formation of breast and prostate origin consists of epithelial cells in which a malignant transformation has occurred. These cells are characterized by disrupted cell cycle, uncontrolled proliferation and accumulation of a large number of mutations. The disrupted cell cycle control is related to change in the expression of cycline dependent kinases (Malumbres and Barbacid, 2005). Their regulatory subunits are named cyclins. The formation of cyclin complexes with their kinase partner regulates the process of cell cycle progression. Different cyclins play a role in the processes of malignant transformation, for example essential role in breast cancer transformation has the cyclin D1. Its role is clarified on the base of studies conducted with experimental mouse models (Casimiro et al., 2013). Typical for tumor formations is the possibility of vascularization and metastasis. In many cases tumor growth depends on the hormonal status. For example, most often the growth of malignantly transformed breast cancer cells is estrogen and/or progesterone-dependent (Lanari et al., 2009) and these hormones are the drivers of breast

tumors progression (Patani and Martin, 2014) because of the progesterone and/or estrogen receptor availability on the cancer cells membranes. However, in many cases malignantly transformed epithelial cells of the breast do not expose estrogen or progesterone receptor but have epidermal growth factor and transforming growth factor receptors. The growth of such cells is continuous in the presence of epidermal growth factor, which in practice makes these types of breast cancer incurable through hormonal deprivation. Transformed cell lines are convenient model systems to study the effect of different treatments on tumor growth and proliferation. For example, the widely used MCF7 is estrogen receptor positive breast cancer cell line isolated from metastatic pleural effusate, characterized by relatively slow growth and expression of normal p53 gene (http://web.expasy.org/cellosaurus/CVCL_0031). The cell line MDA-MB-231 of metastatic epithelial origin is also isolated from pleural effusate. This line does not express estrogen and progesterone receptors but expresses epidermal growth factor receptor and transforming factor receptor, making it much more aggressive; there is also a mutated p53 gene (http://web.expasy.org/cellosaurus/CVCL_0062). Both cell lines are actively used in studies related to the search for new therapeutic approaches in a metastatic breast cancer medication. In the context of the differences in their genetic background, a comparison of the effects of different substances on both cell lines allows for elucidation of a number of mechanisms associated with the operation of various signaling pathways in metastasis, and for inhibiting tumor growth and activation of apoptotic pathways in breast cancer. The growth of prostate cancer cells is androgen dependent (Bennett et al., 2010). This androgen dependence is described in different models, summarized in several mechanisms and used in anti-tumor therapy - depriving the cells of the hormone necessary for their growth. The prostate cancer cells can possess castrate resistant phenotype. Castrate-resitant phenotype is a possibility of prostate cancer cells to grow in case of low androgen hormone concentration. This means that the androgen deprivation therapy is ineffective for these types of cancer. One possible mechanism for acquisition of castrate resistant phenotype is a case of loss of functional androgen receptor. For these types of prostate cancers the hormone replacement therapy becomes ineffective. Good model systems of prostate cancer are the cell lines PC3 and LnCap. PC3 cell line is metastatic cell line of bone origin. It has an aggressive phenotype because of the facts it is androgen unresponsive and $p53^{-}/p53^{-}$. These facts favour the study of the molecular processes related to the hormone deprivation-resistant phenotype and possible mechanisms of the treatment independent of p53 pathway. The cell line LnCap is metastatic one of lymph node origin with androgen responsiveness and normal p53 status. Comparative studies on the response of two cell lines to new substances give possibilities to establish targets for therapy and new methods for cancer treatment.

Classic treatment of cancer is considered to involve invasive surgeries of the affected areas, followed by chemotherapy, and/or radiotherapy.

All of those are linked to side effects for patients. In about 70% of the breast cancer patients is observed dependency of the tumor growth on the female sex hormones estrogen and progesterone (Masood, 1992). However, cancer cells could change their characteristics and become resistant to hemotherapeutic drugs. The mechanisms of drug resistance development are related to many processes: change in drug metabolism and/or procarcinogen metabolism, decreased drug activation, drug inactivation, increased DNA damage repair, drug target alterations, cell death inhibition and an initiation of epithelial-mesenchymal transition (Housman et al, 2014). Frequent acquisition of drug resistance determines the constant search of new possibilities for cancer treatment. A therapy blocking the estrogen/progesterone receptors is applied for breast cancer, in addition to the conventional treatment methods, which limits the growth of such hormone-dependent cancer cells. The remaining of the breast cancer cases are tumors where the cells lack such receptors and are therefore characterized with faster growth, stronger invasiveness, and more inclination towards metastasis development (Lumachi et al., 2013). This is the reason why new treatments are constantly being searched in addition to the conventional therapy. Detailed elucidation of the mechanisms of tumor growth, invasion and metastasis is necessary as a basis to help us locate suitable targets in cancer cells. It is also essential that the new substances show minimal effect on healthy cells in patients' organisms and a few probability for cells to develop drug resitance.

2. THE ROLE OF PROTEASES IN CANCER GROWTH, INVASION AND METASTASIS

There is accumulated evidence about the role of proteases in cancer transformation, development, metastatic process and epithelial-mesenchymal transition. Proteases as a whole have an important role in both the normal functioning of the body's cells, and the development of many diseases, including cancer. An impressive set of proteases and their homologues is encoded by the human genome - about 569, with representatives of five major catalytic classes: metalloproteases -194, serine – 176, cysteine – 150, threonine - 28 and aspartate proteases - 21 (Rakashanda et al., 2012). Proteolysis participates in the whole process of cancer development, progresion and metastasis (Castro-Guillén et al., 2010). With a proven role in the process of tumor growth, invasion, angiogenesis and metastasis are basically three classes of human proteases – serine-, cysteine- and metalloproteases (Rakashanda et al., 2012, Aggarwal and Sloane, 2014, Kryza et al., 2016). Serine proteases play an important role in the processes of growth and differentiation in normal cells. Tissue kallikreins, a family of 15 secreted serine proteases with chymotrypsin fold, are implicated in the regulation of homeostatic functions. Their expression is estrogen/progesterone dependent and an increased expression of kallikreins is detected in many cell lines of breast cancer (Diamandis and Yousef, 2002). Prostate-specific antigen (human kallikrein 3) is widely used as a tumor marker for screening, diagnosis and monitoring of prostate

cancer (Mueller-Lisse et al., 2002). It is established that some kallikreins facilitate the progression and metastasis of cancer cells due to their increased activity, which leads to accelerated degradation of the extracellular matrix components. Kallikreins could also regulate by proteolysis the amount of certain proteins involved in signaling pathways and thus could mediate cell proliferation, growth and metastasis. For these reasons, kallikreins are a potential target for treatment of tumors (Kryza et al., 2016). Another serine protease - hepsin, located on the cell surface, cleaves extracellular substrates and contributes to the proteolytic processing of growth factors. Hepsin plays an essential role in cell growth and the maintenance of cell morphology (Torres-Rosado et al., 1993). Hepsin is involved in the progression and metastasis of breast cancer. Increased expression of hepsin is associated with tumor stage, metastasis to lymph nodes and with the presence of estrogen and progesterone receptors (Xing et al., 2011). The ubiquitin-proteasome system controls multiple signaling and regulatory pathways and its inhibition can lead to efficient tumor suppression (Frankland-Searby and Bhaumik, 2012). The ubiquitin proteasome pathway is required for targeted degradation of many short-living proteins in eukaryotic cells. Among proteasome targets are cell cycle regulatory proteins as well as proteins unable to fold properly within the endoplasmic reticulum. Ubiquitination is a complex ATP dependent process in which participate 3 types of enzymes - ubiquitin activating enzyme (E1) which forms a thio-ester bond with ubiquitin (a highly conserved 76-amino acid protein); ubiquitin conjugating enzyme (E2) which accepts the activated ubiquitine and transfers it to one of the many ubiquitin ligases (E3s). E3 then ransfer the ubiquitin from the E2 cysteine to a lysine residue on the target protein, followed by the formation of a covalent isopeptide bond between the carboxy-terminus of ubiquitin and a lysine residue on the substrate protein. Numerous E3 ligases provide the specificity of the process. Monoubiquitination has a role in endocytosis, as well as changes in subcellular protein localization and trafficking, while polyubiquitination is required for targeting a protein for degradation by the proteasome. Ubiquitination is a reversible process, deubiquitinating enzyme catalyze the removal of ubiquitin from target proteins (Amm et al., 2014). The proteasome is a threonine type protease possessing trypsin-like, chymotrypsin-like and caspase-like activities, with localization in cytoplasm and nucleus. Inhibition of the proteasome by protease inhibitors triggers apoptosis (Mehdad et al., 2016). Ubiquitin-proteasome pathway plays an essential role in protein maturation, in cancer growth, metastases formation and cancer spread. The role of the ubiquitin in cancer development is as important as that of phosphorylation/dephosphorylation. Each of two is a part of protein modification system and a change in their regulation play a key role in many signaling pathways and cell regulatory events in cancer (Pal and Donato, 2014). Protein deubiquitination is implicated in the regulation of critical pathways related to cancer development. Such types of processes are the internalization and degradation of receptor thyrosine

kinases, localization and activity of different types of intermediates, gene transcription, cell cycle progression (Stegmeier et al., 2007), apoptosis (Priolo et al., 2006), chromosomal translocation and DNA damage repair (Cummins et al., 2004). Within the group of cysteine proteases, some cathepsins have been reported to accumulate in cancer cells, and in particular cathepsin B - lysosomal protease with papain type folding. In malignantly transformed cells changes in the location of cathepsin B are reported - secretion in the extracellular space instead of the lysosomal compartment (Aggarwal and Sloane, 2014). Cathepsin B facilitates metastasis directly, by digestion of the major components of the extracellular matrix, and indirectly, by degradation of the tissue inhibitors of metalloproteases, thus involving this class of proteases in the metastatic process, too. The identification of secreted proteins in tumour interstitial fluid sheds light on the complex proteolytic network in breast cancer secretome (Gomes-Auli et al., 2016). The lysosomal cathepsin protease has an essential role in cellular remodeling during the process of epithelial mesenchymal transition, directed by TGFβ signaling which is a key mediator of cancer progression (Kern et al., 2015). Destruction of the extracellular matrix components in the vicinity of a tumor is particularly well expressed in tumors of epithelial origin, including breast cancer. Another type of proteases with altered expression and prognostic significance in various types of breast cancer are the cytoplasmically localised calpains, which are calcium-dependent cysteine proteases involved in many cellular functions such as remodeling of the cytoskeleton, cell signaling, apoptosis (Storr et al., 2016). The multitude of proteases perform their functions in a coordinated manner, besides, they interact with the kinase phosphatase signaling pathways. Thus, proteases form proteolytic cascades/ networks; they also can partially compensate for the lack of any of them (Mason and Joyce, 2011).

3. REGULATION OF TUMOUR PROTEASE ACTIVITY BY PROTEASE INHIBITORS

Due to the nature of their action, proteases are synthesised as pre-pro-enzymes, are subjected to maturation and modifications in a multistep process involving other proteases or autocatalytic mechanisms, and protease activity is subjected to strict control by endogenous protease inhibitors specific for a given class/type of proteases. Key regulators of proteases functions are protease inhibitors (PIs). In the process of cancerogenesis and metastasis, abnormal ratio between proteases and endogenous PIs in cancer cells is reported, associated with decreased expression/activity of certain PIs (Storr et al., 2016, Dabiri et al., 2016). The efforts of many researchers are focused on seeking means to compensate for the insufficient availability of PIs by applying external ones for the treatment of cancer. Besides, the use of PIs in experimental models gives possibilities to clarify the mechanisms of processes related to cancer development.

Protease inhibitors are a group of substances, which in recent years has been the subject of an intense research (Shamsi et al., 2016). Their ability to inhibit proliferation of tumor cells has

been shown in a number of studies both in *in vivo* (animal models) and *in vitro* conditions (cell lines). Research on the use of synthetic protease inhibitors as anti-tumor drugs dates back several decades. Such studies have been made initially with inhibitors tested for treatment of patients with acquired immunodeficiency (Castro-Guillén et al., 2010). The most well studied as concerning the mechanism of action is the typical synthetic protease inhibitor nelfinavir. There are some studies on nelfinavir analogs as well as on other synthetic protease inhibitors like ritonavir and saquinavir. They are all inhibitors of the HIV proteases, for which suppressory action on the regulated intra-membrane proteolysis has been found (Guan et al., 2015). Nelfinavir inhibits two serine proteases in the endoplasmic reticulum - S1P and S2P, which are necessary for the maturation of SREBP1 protein, a transcription factor regulating lypogenesis and cholesterol synthesis (Brown and Goldstein, 1997) as well as for the maturation of other proteins, necessary for proper folding of proteins in the endoplasmic reticulum (Koltai, 2015). Nelfinavir inhibits proliferation and induces apoptosis through reduced expression of the Bcl2 anti-apoptotic protein, which is an indication for the involvement of the mitochondrial (internal) pathway of apoptosis. The accumulation of immature form of S2P serine protease in the endoplasmic reticulum leads to stress which causes cell death as a result of fatty acid synthesis inhibition in the cells. The above described mechanism leads to cell death by endoplasmic reticulum stress, growth arrest of the cells, increased frequency of apoptosis, and decreased invasion of cancer. Sequinavir shows antiproliferative effect and anti-invasive activity against neuroblastoma cells. These effects are mediated by NfkB-decreased expression (Timeus et al., 2012). It was established that sequinavir and ritonavir reduce MMP2 and MMP9 inhibiting cell invasion and growth of in cervical intraepithelial neoplasial cells (Barillari et al., 2012). These facts are proven on cancer cell lines of different origin, on cancer mouse models and in preclinical case studies. The antitumor properties of the synthetic protease inhibitors are obvious but they have a number of side effects. Undesirable changes in immune functions related to the effect on the differentiation of monocytes and dendritic cells, as well as multi-resistance to standard anti-cancer agents have been established (Crum-Cianflone et al., 2009). Therefore, the efforts of scientists are directed to search ways for medication with possible minor side effects. Protease inhibitors of plant origin can be an alternative or an addition to the treatment with synthetic inhibitors.

4. PLANT PROTEASE INHIBITORS AS NATURAL CANCER-PREVENTING NUTRITION INGREDIENTS AND THEIR ANTICANCER POTENTIAL

Protease inhibitors of plant origin have the potential to be an alternative or supplement to the treatment with synthetic drugs. The interest in them originates from the application of plant PIs in prevention of carcinogenesis (Kennedy, 1998). It has been established that a diet rich in seeds of legumes and cereals (with a high content of PIs of plant origin) leads to a

significant reduction in the probability of occurrence of a number of cancers such as colon cancer, breast cancer, prostate cancer (Rakashanda et al., 2012). Plants posess a large spectrum of proteinaceous protease inhibitors, which serve them both for defense against proteases secreted by phytopathogens and pests and for regulation of endogenous proteolytic activity within plant cells (Leo et al., 2002, Habib and Fazili, 2007). Seeds of monocotyledonous and dicotyledonous plants are enriched in PIs, which could comprise about 5–10% of the total water soluble protein content (Srikanth and Chen, 2016). Protease inhibitors in storage organs (seeds and tubers) exert triple function – as defense proteins, as storage proteins, and as regulators of proteolytic activity. As in other living organisms plant PIs, by control of endogenous proteases, perform essential regulatory functions on cell growth and differentiation, cell cycle, misfolded protein response, programmed cell death, as well as on developmental processes (Vaseva et al., 2012).

Plant PIs are relatively low molecular weight proteins classified in distinct groups, following different criteria. According to their specificity to the catalytic type of protease, inhibitors of all major classes of proteases are found in plants, predominately against serine proteases, followed by inhibitors of cysteine and metalloproteases (Salvesen and Nagas, 1989; Rawlings, 2010, Shamsi et al., 2016). In plants, typical representatives of PIs against serine proteases are the Kunitz type inhibitors (KTI) with moleculas mass 18-22 kDa, Bowman-Birk type inhibitors (BBPI, 7-9 kDa), serpins (39-43 kDa), and against cysteine proteases – the phytocystatins with moleculas mass 11-23 kDa, as well as multidomain cystatins (Habib and Fazili, 2007). Some PIs in plants possess broader specificity against more than one class of proteases, for example KTIs could inhibit not only serine proteases from S1 family, but also cysteine proteases from C1 family and the aspartic potease cathepsin D (Habib and Fazili, 2007). According to the mechanisms of their inhibitory action, two general mechanisms are considered - irreversible "trapping" reactions and reversible, tight binding reactions between PI and the respective protease (Rawlings, 2010). Trapping reactions are typical for the "suicide" PIs serpins which bind covalently to the protease`s active site and undergo a conformational change, thus blocking it permanently. KTI and BBPI follow the classical inhibitory mechanism in a substrate-like manner, whereas phytocystatins could exert inhibitory action by binding to exosites (Habib and Fazili, 2007). In the MEROPS database both proteases and protease inhibitors are grouped in families and clans on the basis of sequence and tertiary structure (protein folds and domains) similarities (Rawlings, 2010). According to this classification, soybean KTI belongs to IC clan, I3 family and presents beta-trefoil fold, whereas BBPI from vigna belongs to IF clan, I12 family and has a knottin fold (Rawlings 2010). The knottin fold contains a disulfide-bonded core that confers remarquable proteolytic resistance and thermal stability of BBPIs (Moore and Cochran, 2012).

Plant PIs against serine proteases, especially the KTI and BBPI families, have been thoroughly studied for their anti-carcinogenesis and anti-metastatic properties in a variety of tumors (Castro-

Guillén et al., 2010). Kunitz PIs have broad spectrum of molecular targets and can act on different signaling molecules, including key proteases in cancer invasion; their action is principally as antiangiogenesis and anti-metastatic agents (Castro-Guillén et al., 2010). Bowman-Birk PIs are considered as most promising PI in cancer research. These inhibitors exert a double blocking action against trypsin- and chymotrypsin-like serine proteases (Kennedy, 1998). It is established that the chymotrypsin inhibitory domain plays a main role in the suppressive effects on carcinogenesis; moreover, an extended effect of BBPIs against radiation-induced transformation after carcinogen exposure has also been shown probably linked with DNA repair mechanisms (Castro-Guillén et al., 2010). Besides, no clinical toxicity or drug allergy for BBPIs has been established.

Bowman-Birk PI is accumulated in high concentrations in the seeds of leguminous plants, presenting variations in quantity and isoinhibitory profile depending on species and varieties (Gonzalez de Mejia and Dia, 2010, Clemente et al., 2013). Particularities in the structure (rigidity of the molecule due to characteristic highly conserved seven intra-molecular disulfide bridges; resistance to heat, to pH extremes, to breakdown by digestive proteases) render BBPIs capable to be transported across the gut epithelium, to reach target tissues and act locally (Clemente et al., 2013). Thus, it is not surprising that a diet rich in seeds of legumes, which are good source of BBPIs, protects the body from malignant transformation. A number of studies have shown the role of individual protease inhibitors of plant origin as antitumor agents. On model systems of cell lines with different origin it is established that the BBPI reduces the survival rate of cancer cells, their proliferation, and in many cases it drives the process of programmed cell death (Magee et al., 2012, Mehdad et al., 2016). However, there are insufficient data on the mechanism of BBPI antitumor effects. Most likely, its action is associated with direct or indirect influence of the proteasome and induction of endoplasmic reticulum stress in metastatic cell lines, but pleiotropic effect on more proteases is also possible. More likely the primary targets would be secretory and/or cell membrane located serine proteases. The mechanism of cell internalisation of plant protease inhibitors is also of interest. At present it is only known that Buckwheat trypsin inhibitor enters the Hep G2 cells by clathrin-dependent endocytosis (Cui et al., 2013).

The inhibitors of cysteine type proteases of plant origin (phytocystatins) also have the potential to be used in research related to their effects on the metastatic potential of tumor cell lines and animal cancer models. Their mechanism of action could be related to inhibition of the lysosomal proteases cathepsin type and of other proteases, involved in the degradation of the extracellular matrix, but other targets could be also possible (Turk et al., 2008).

The therapeutic potential of plant protease inhibitors for the treatment of cancer is still in the process of clarification (summarized in Shamzi et al., 2016). In addition, plant protease inhibitors could be used as tools (by blocking certain proteases) to study the mechanisms of tumor growth, invasion, angiogenesis, and metastasis.

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