Abstract. Calpains belong to a family of important calcium-dependent cysteine proteases. They are involved in intracellular processes including cytoskeleton disorganization and substrate proteolysis. They also enhance apoptosis and cell to cell adhesion. Calpains demonstrate also a mechanosensory function in neoplastic and malignant cells due to their implication in mechanoptosis. This is a specific type of apoptotic death induced by strong external mechanical stimuli. Anti-cytoskeleton rigidity inhibition strategies based on calpain induction lead to increased apoptosis of tumor transformed cells. Elevated intracellular calcium concentration mediated by specific receptors and channels activates calpains. In the current molecular review, we explored the role of calpains in calcium-dependent signal transduction pathways in breast adenocarcinoma in conjunction with novel agents that activate their important anti-tumor functions.

Correspondence to: Evangelos Tsiambas, MD, MSc, Ph.D., Cytologist, 17 Patriarchou Grigorou E' Street, Ag, Paraskevi, 153 41 Athens, Greece. Tel: +30 6946939414, e-mail: tsiambasecyto@yahoo.gr

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Regulation of intracellular calcium concentration plays a significant role in securing biochemical cell homeostasis. Ca²⁺ activates a variety of crucial enzymes leading to increased signal transduction to the nucleus. Modified ion channels on the cell membrane are responsible for Ca²⁺ influx from the extracellular space due to an electrochemical gradient regulation mechanism (1). In fact, Ca²⁺ is involved not only in ion channel activation, but also in indirect intracellular signal transduction cascade mediated by G protein and tyrosine kinase cell receptors as a repetitive ubiquitous messenger (2). Endoplasmic reticulum and mitochondria participate in intracellular release of Ca²⁺. Additionally, phospholipase C-dependent pathway regulates intracellular Ca²⁺ transduction and concentration (3). Intracellular Ca²⁺ is involved in critical cell functions including muscle normal growth and contraction, cellular motility provided by membranous cilia or flagella, embryonic development of neuronal tissues, maturation, activation, and synaptic signal transmission, cytoskeleton stability and flexibility, cell proliferation, and apoptosis are critical (4). Furthermore, Ca²⁺ homeostasis enhances DNA repair procedures and hormone secretion, whereas its deregulation induces metastatic potential, epithelial-mesenchymal transition (EMT), and angiogenesis in cancerous tissues (5).

A variety of Ca²⁺-dependent proteins – including transmembrane glycoproteins and proteases – are implicated in cell-to-cell adhesion. Among them, calmodulins, cadherins, integrins, selectins, calpains [calcium activated neutral proteases (CANPs)] are the most recognizable (6). In the current review, we explored the role of CANPs...
deregulation in breast carcinoma (BrCa) biochemical substrate and present novel and promising CANP inhibitors for oncological targeted treatment approaches.

**CANPs: Protein Structure and Functions**

The CANP superfamily (C2 protease family) comprises sixteen members of proteolytic enzymes. CANPs are Ca^{++}-dependent non-lysosomal cysteine proteases. CANPs form a proteolytic system that consists of enzymes, the calpain small regulatory subunits, and the corresponding endogenous inhibitors, mainly the caplastatin (7). In fact, micro (μ) - and milli (m) - CANPs represent the initial, precursor family members. CANP functional heterodimers comprise two main domains: the large and the small, respectively. Ubiquitously expressed micro- and milli-CANPs form the previous described heterodimers. More specifically, CANPs 1(11q13.1)/2(1q41)/4(19p13.1) are large subunits, CANP1/2/4 are small regulatory subunits, and CANPs 3-14 (excluding number 4) are large catalytic and proteolytic domains. Moreover, CANPs proteolyze their substrates. In contrast, other proteolytic intracellular proteins such as caspases degrade their corresponding substrates (8). Interestingly, at the biochemical level, Ca^{++}-mediated CANP phosphorylation and dephosphorylation equilibrium modifies their activity toward the corresponding substrates. CANPs are implicated in several significant cell functions including cell cycle phase progression, cell motility, and functional enhancement and fusion, especially in specific types of cells such as myoblasts, neurons, and dendrites (9). Interestingly, CANP2 acts as a protease that regulates focal adhesion and talin cleavage. They are also involved in apoptosis and necrosis, clotting development inside blood vessels, and hypoxia regulation in the cases of increased platelet concentration and over activation (10).

**CANPs in BrCa Mechanoptosis**

Tumors -especially malignant ones- are characterized by an excessive cell proliferation that leads to an altered, non-geometrical transformation of growth (11). In conjunction, neoplastic and malignant cells lack specific sensors that secure cellular rigidity and shape stability (12). This is a significant disadvantage compared to normal cells, which can resist to cellular rigidity and shape stability (12). This is a significant disadvantage compared to normal cells, which can resist to external mechanical pressure. The current mechanism is detected in a variety of BrCa types including the inflammatory one. In these BrCa types, CANPs influence the generation of the E-cad/NTF1 complex (15, 16).

Concerning BrCa onset and progression, there is strong evidence for the involvement of specific Ca^{2+} channels that are modified by CANPs. A study group explored the role of ORAI3 Ca^{2+} channels in BrCa cell cultures to examine their impact on chemotherapy resistance combined with their tendency to migrate (tissue to tissue metastasis). They detected decreased CANP levels when ORAI3 Ca^{2+} channels lost their function (17). Additionally, they identified a specific interaction between ORAI3 and focal adhesion kinase (FAK), which is significant for the actin-related cytoskeleton stability. Orai3 – CANP alterations were found to be critical for cancerous cell-to-cell adhesion and migration potential. Similarly, another study focused on the conjunction between CANPs and actin-binding proteins of the cytoskeleton and their impact on metastasis and tissue-to-tissue invasion in BrCAs (18). They analyzed the role of CANPs and proteasomes in the remodeling process of the internal cell microenvironment that regulates tumor progression and even lymphogenous metastasis in BrCa. Furthermore, another study reported that activation of proteasomes and CANPs alterations increased lymphogenous metastases in BrCa and lung cancer patients (19). Another study showed a relation between isoform 2 of the glycolytic enzyme hexokinase (HK2) and Ca^{2+} -mediated CANP activation (20). More specifically, they observed that HK2 displacement releases high levels of CANP. This mechanism triggers the apoptotic cell death due to extensive mitochondrial depolarization. Interestingly, CANPs demonstrate different expression levels in triple-negative breast cancer (TNBC) cases detected in women with different demographic/genetic characteristics. A research group explored the potential differences in the expression of CANPs, pyrimidine-based pathways, and innate immune signaling pathways (21). They detected significant alterations in these pathways by comparing the origin (African American to European American) of the examined BrCa cases. A more aggressive phenotype was observed in the African–American category. Similarly, another study showed that CANP1 functional over activation inside the endoplasmic reticulum led to sensitization and induction of cisplatin-induced apoptosis in breast cancer cell lines (22).

Mechanisms of mechanosensitive cell adhesion and migration in malignant neoplasms including BrCAs are under investigation. CANPs have been found to be involved in these mechanisms by interacting with the epidermal growth factor receptor (EGFR) and the integrin-dependent signaling transduction pathways (23). In fact, the integrin α6 isoform-
mediated pathway interacts with the extracellular matrix (ECM). Furthermore, EGFR phosphorylation induces CANP over-expression in breast cancer cells, preventing metastasis. Interestingly, β-catenin, E-cadherin, talin-1, and p-120 represent critical targets for CANPs. A study showed that CANP1/2 isoforms cleave cell adhesion molecules allowing the migration of BrCa cells. Interestingly, this mechanism is observed also in normal procedures such as the post-lactation period (24). In addition, altered expression of some members of the CAPN superfamily (CANP1/2/4) and their inhibitor – calpastatin – seems to negatively influence the biological behavior and response rates to chemotherapeutic agents such as platinum in other significant female malignancies including ovarian carcinoma (25). Also, CANP2 induces cleavage and activation of LIM Kinase-1 (LIMK1), and this action is critical for the potential involvement of CANPs in nuclear processes, such as mitosis. Interestingly, a study group observed CANP2 nucleolar localization during interphase (26). Furthermore, another study analyzing BrCa cell cultures (MCF-7 lines), showed that CANP activation leads to an excessive EMT due to increased fibronectin expression (27).

Referring to specific CANPs in BrCa, CANP1 over-expression was shown to be related with poor relapse-free survival in a series of cases treated with trastuzumab-based targeted therapeutic regimens (28). Similarly, increased CANP1 protein production was found to be associated with lymph node metastasis in TNBC cases (29). Additionally, the relation between CANP1 and androgen receptor (AR) in BrCa is under research. A study group co-analyzed their expression profiles in a series of cases concluding that low CANP1 expression was correlated to shorter 5-year disease-free survival, whereas AR levels were not statistically significantly different in the examined cases (30). It should be also mentioned that CANP1 is protected from proteolytic degradation and functional inactivation by a modified agent, the hcast 3-25 calpastatin (31). Calpastatin and calpeptin are the main inhibitors of CANPs that reduce their mitochondrial activity. In carcinomas, including BrCa, they are involved in cancer cachexia. In fact, they prevent skeletal myoblasts’ apoptosis (mechanoptosis) mediated by CANPs (32). Furthermore, CANPs1/2 demonstrate a critical activity in HER2 positive BrCas. An experimental study analyzed their co-expression in transgenic mouse models. They observed that their down-regulation reduces carcinogenesis sensitizes cancerous tissues to specific targeted therapeutic agents such as lapatinib and doxorubicin (33, 34). Especially, CANP2 seems to modify nuclear forkhead box protein P1 (N-FOX1) expression levels in BrCas (35). Additionally, it is also co-expressed with AKT/m TOR proteins. Interestingly, two other calpains, CANP9 and CANP10, are implicated in mesenchymal transition-based myofibroblast embryogenesis, development, and differentiation. They also reduce HER2 expression in sub-sets of patients (36, 37). Furthermore, in HER2 positive BrCa cases, co-inhibition of CANP1/2 and heat shock protein-90 (HSP90) leads to attenuation of breast carcinogenesis (38). Concerning the role of CANPs inhibition in malignancies, a study group explored several mechanisms that are involved in multidrug resistance. They used membrane-derived microparticles in a series of BrCa cell lines (MCF-7 and MCF-7/Dx) (39). They reported a significant vesiculation in the examined malignant cells due to CANPs over-activation in malignant cells. In addition to this, it is important to mention that CANP1/2 over-expression decreases cancerous stem cells potential in breast carcinoma tissues, as shown in an experimental study by analyzing MCF7 and MCF10AT cell cultures (40). For this reason, thapsigargin-based CANP1/2 activation could be a significant targeted therapeutic approach.

In conclusion, CANPs is a significant calcium-dependent protein family involved in cytoskeleton disorganization and substrate proteolysis. They enhance a specific type of apoptotic death –mechanoptosis- following severe external mechanical stimuli and in cancer-related cachexia. Anti-cytoskeleton rigidity inhibition strategies based on CANPs induction led to increased apoptosis in tumor cells. Elevated calcium intracellular concentration mediated by specific receptors and channels activate CANPs. Concerning BrCa, CANPs are deregulated affecting the corresponding calcium-related signal transduction pathways and could be crucial targets for novel agents that activate their important anti-tumor functions.

**Conflicts of Interest**

The Authors have no conflicts of interest to declare in relation to this study.
Authors’ Contributions

GM and ET: design of the study, article writing, DP, DS, and CD: academic advisors: EF, SM, LM, DD, and PP: collection and management of references and published data. All Authors read and approved the final article.

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