Atrial Natriuretic Peptide: A Magic Bullet for Cancer Therapy Targeting Wnt Signaling and Cellular pH Regulators

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Abstract: Atrial natriuretic peptide (ANP) is a cardiac hormone playing a crucial role in cardiovascular homeostasis mainly through blood volume and pressure regulation. In the last years, the new property ascribed to ANP of inhibiting tumor growth both in vitro and in vivo has made this peptide an attractive candidate for anticancer therapy. The molecular mechanism underlying the anti-proliferative effect of ANP has been mainly related to its interaction with the specific receptors NPRs, through which this natriuretic hormone inhibits some metabolic targets critical for cancer development, including the Ras-MEK1/2-ERK1/2 kinase cascade, functioning as a multikinase inhibitor. In this review we summarize the current knowledge on this topic, focusing on our recent data demonstrating that the antitumor activity of this natriuretic hormone is also mediated by a concomitant effect on the Wnt/β-catenin pathway and on the pH regulation ability of cancer cells, through a Frizzled-related mechanism. This peculiarity of simultaneously targeting two processes crucial for neoplastic transformation and solid tumor survival reinforces the utility of ANP for the development of both preventive and therapeutic strategies.

Keywords: Antitumor effect, atrial natriuretic peptide, cardiac hormones, corin, frizzled, mechanism(s) of action, NHE-1 inhibition, pH regulators, target therapy, tumor microenvironmental pH, Wnt pathway.

INTRODUCTION

Atrial natriuretic peptide (ANP) is a 28aa peptide that belongs to a family of cardiac and vascular-derived hormones playing a crucial role in cardiovascular homeostasis mainly through blood volume and pressure regulation [1, 2]. Besides its role in cardiovascular homeostasis, in the last years a new property of ANP has been discovered, namely, its ability of inhibiting tumor growth both in vitro and in vivo [3-9]. For this property, this hormone peptide has been proposed as an attractive candidate for innovative anticancer therapy [10, 11]. The anti-proliferative efficacy of ANP has been extensively demonstrated on human pancreatic adenocarcinoma, as well as on human breast, prostate, colon, renal, ovarian, small cell and squamous cell lung cancers and on other tumors [3-9]. Until now the molecular mechanism underlying the anti-proliferative effect of ANP is not yet completely understood, even if many modalities of action have been proposed. This review aims to summarize the current knowledge on this topic, focusing on recent data sustaining that this natriuretic hormone targets the Wnt/β-catenin pathway and the pH regulation ability of cancer cells, two process crucial for solid tumor survival.

ANP MATURATION: THE ROLE OF CORIN

ANP is stored, as pro-peptide, in cytoplasmic dense granules of cardiomyocytes and is released into the blood stream upon atrial stretch resulting from elevated blood pressure, thus decreasing pressure overload and reducing cardiac hypertrophy [1, 2]. Like most of signaling molecules, ANP is synthesized as acid inactive precursor (pro-ANP), that contains other three natriuretic peptides, the long-acting natriuretic peptide (LANP), the vessel dilator (VSDL), and the kaliuretic peptide (KP) [12] (Fig. 1A). ANP is secreted from cardiomyocytes and proteolytically cleaved by the membrane-associated protease Corin, that converts it to the mature active peptide [13, 14] (Fig. 1B and C). Corin mRNA and protein, that are highly expressed in cardiomyocytes [15, 16], have been also detected in cancer cell derived from osteosarcoma, leiomyosarcoma, endometrial carcinoma, and small cell lung cancer [15, 17]. The extracellular region of Corin, which contains the structural domains essential for pro-ANP processing [13], includes two Frizzled-like cysteine-rich domains (Fz1 and Fz2), which are members of the transmembrane receptor for Wnt signaling proteins [18] (Fig. 1C). As demonstrated by Knappe et al. [13] Frizzled 1 domain and LDLR repeats 1-4 are important structural elements through which Corin recognizes its physiological substrate, pro-ANP (Fig. 1C). In particular, Fz1 domain is involved in the interaction of Corin with pro-ANP. These Authors proposed that, upon release from the dense granules of cardiomyocytes, pro-ANP binds to Fz1 and LDLR repeats 1-4, is cleaved by the catalytic domain and releases the mature and biologically active ANP (Fig. 1C).

MECHANISM(S) OF ACTION OF ANP: SPECIFIC RECEPTORS AND METABOLIC TARGETS

ANP exerts its antitumor effect by significantly inhibiting DNA synthesis but not by inducing apoptosis [3]. It has been
recently demonstrated, for prostate, lung, and kidney cells, that ANP - as well as the other three pro-ANP-derived peptides - exerts its cytotoxic effect exclusively on cancer cells sparing cells derived from healthy individuals [19]. Most of the molecular mechanisms proposed for the anti-proliferative effect of ANP and the other heart hormones LANP, VSDL and KP, have been resumed in a recent review by Vesely [11]. A potential interaction between ANP and its specific receptors NPR-A, NPR-B and NPR-C, which are expressed at different levels on tumor cells [3-9, 20] has been proposed. After binding to their respective receptors on the tumor cells, all the four natriuretic peptides inhibit some metabolic targets including the GTPase RAS [21], the MEK1/2 kinase [22] and the extracellular signal-regulated kinase (ERK) 1/2 [23]. In addition to its ability of inhibiting the Ras-MEK1/2-ERK1/2 kinase cascade, functioning as a multikinase inhibitor, it has been recently reported that ANP (as well as the other three natriuretic hormones) is able to decrease, in tumor cells, the levels of vascular endothelial growth factor (VEGF) and its main receptor VEGFR2 [24], and of the Signal Transducer and Activator of Transcription 3 (STAT 3) [25], that are three molecular targets useful for cancer therapy [26-28]. Moreover, owing the observed localization of ANP in the nuclear compartment of human pancreatic adenocarcinoma cells, a direct interaction of the peptide with growth-promoting hormones in the nucleus has been hypothesized [29].

**INNOVATIVE TARGETS FOR ANTITUMOR ACTIVITY OF ANP**

Besides the above mentioned modalities of action correlated to the interaction of ANP with the specific receptors NPRs, it has been recently demonstrated in our laboratory that the antitumor activity of this natriuretic hormone is mediated by a simultaneous effect on the Wnt/β-catenin pathway and on the pH regulation ability of colorectal cancer cells [30], through a Frizzled-related mechanism. This innovative aspect of ANP anticancer efficacy, is partially sustained by the results obtained by Vesely and collaborators, that demonstrated its effect on some components of the Wnt signaling [31].

**The Wnt/β-catenin Signaling Pathway in Cancer Cells**

In the last years, great attention has been given to some signaling pathways which deregulation or constitutive activation have been demonstrated to have a role in cancer insurgence and progression. These pathways could be of interest for therapeutic perspectives, because targeting them may contribute to prevent tumorigenesis or achieve tumor reversion. In particular, numerous studies demonstrated the key role of deregulation or constitutive activation of the Wnt signaling pathway in the initiation and progression of different forms of human cancer [32, 33]. Hence, several molecular components in the Wnt signaling pathway have been pro-
posed as novel targets for cancer therapy [33-35]. As schematized in (Fig. 2), in non-transformed cells, β-catenin, the most important mediators of the Wnt signaling [33, 34], exists in a cadherin-bound form that regulates cell-cell adhesion, and the β-catenin excess, not segregated by E-cadherin on the cell membrane, is rapidly phosphorylated by glycosgen synthetase kinase-3β (GSK-3β) in the adenomatous polyposis coli (APC)/axin/GSK-3β destruction complex and is subsequently degraded by the ubiquitin-proteosome pathway. Conversely, in tumor cells, Wnt ligands, such as Wnt3a and Wnt1, interact with members of the Frizzled (Fz) family of serpentine receptors and with LRP5 or LRP6 co-receptors. The binding of Wnts to Fz leads to activation and membrane recruitment of the phosphophosphoinositide Dishevelled (DHS). Activated DHS inhibits the destruction complex, by recruiting axin at the plasma membrane and inducing the Akt-mediated inactivation of GSK-3β via Ser9 phosphorylation [36], and causes β-catenin stabilization and cytosolic accumulation. Stabilized β-catenin enters the nucleus, where it acts as a co-activator for TCF/LEF-mediated transcription and ultimately modulates cell proliferation, survival and differentiation. Inactivating mutations of APC or stabilizing mutations of β-catenin, leading to constitutive activation of the Wnt/β-catenin pathway, have been recovered in various cancers [32, 33, 37].

**ANP Affects the Wnt/β-catenin Signaling Cascade**

The presence in the extracellular region of Corin of the Frizzled-like cysteine-rich domains Fz1 and Fz2, involved in the interaction of Corin with pro-APN (Fig. 1C), supports the hypothesis that ANP affects directly or indirectly the Wnt signaling cascade. We have recently demonstrated that ANP exerts its anti-proliferative activity by inhibiting the Wnt/β-catenin signaling cascade in rat and human colorectal cancer cells that exhibit the constitutive activation of the canonic Wnt pathway [30]. Specifically, we demonstrated that ANP i) induces a redistribution of β-catenin from nuclear and cytoplasmic compartments to cell-cell junctions, ii) stimulates the degradation of stabilized β-catenin at the destruction complex and iii) affects the transcription of some Wnt pathway target genes including c-Myc, Cyclin D1 and E-cadherin. We also showed that the ANP-induced decrease of β-catenin in the nuclear compartment was preceded by an early translocation of the onco-suppressor APC to the nucleus, and that APC and β-catenin co-localized firstly in the nucleus and subsequently in cytoplasm [30]. This strongly suggested that, under ANP action, APC might participate in the β-catenin nuclear export toward the cytoplasmic “destruction complex”, through its described shuttling function [38].

The ANP-induced effect on the Wnt/β-catenin signaling seems to involve a Frizzled receptor-mediated mechanism relying on a Wnt receptor/ANP direct interaction. This is supported by the co-localization of ANP and the Frizzled receptor observed on membrane of treated cells as well as by the evidences demonstrating the ability of ANP and Wnt1a in competing and displacing each other from the binding receptor [30]. Our results were sustained by data obtained by Vesely and collaborators, demonstrating that ANP and the other natriuretic hormones LANP, VSDL and KP are able to decrease the concentration of β-catenin up to 88% in human pancreatic cancer cells, up to 83% in human colorectal adenocarcinoma cells, and up to 73% in human renal adenocarcinoma cells [31]. The same Authors also reported that, in pancreatic, colorectal and renal cancer cells, all the four cardiac hormones reduce the concentration of the Wnt pathway activator WNT-3a [31] and decrease the levels of the Secreted Frizzled-related protein-3 (sFRP-3) [39], a secreted glycoprotein that affects Wnt signaling and that has been linked to tumor promotion in different types of cancers [40].

**Cellular pH Regulators and Cancer**

There is growing evidence sustaining the role of tumor cell microenvironment in cancer progression and sensitivity to chemotherapy. In particular, acidic tumor microenvironment has been reported to have a role in cancer cell proliferation [41], invasion and metastatization [42, 43] and in resistance to chemotherapy and radiotherapy [44, 45]. In growing tumor, continued cell proliferation leads to extracellular acidification due to a multitude of factors including poor blood perfusion, low oxygen availability, increased metabolism of glucose and production of metabolic acids, such as lactic acid [46, 47] (Fig. 3A). Extracellular acidosis has the properties to enhance tumor invasiveness, by the degradation of the extracellular matrix [48], and to reduce immune surveillance, by inhibiting the activity of natural killer (NK) cells [49] and the cytolytic activity of cytotoxic T lymphocytes [50]. Thus, preservation of extracellular acidosis is crucial for tumor progression and malignancy. Cancer cells survive and adapt to extracellular acidic stress by over-expressing on cell surface various pH regulator molecules, which avoid cytosolic accumulation of toxic H⁺ ions and contribute to maintain a balance between the extracellular (pHₑ) and intracellular pH (pHᵢ) (Fig. 3A). Among the pH regulators, Proton Pumps (PP) and Na⁺/H⁺ Exchangers (NHE) have been recently proposed as promising molecular targets for cancer chemotherapy [51, 52]. PP or NHE inhibitors, by decreasing the activity of the specific pH regulators, cause accumulation of toxic H⁺ ions in the intracelluar compartments that ultimately results in inhibition of tumor growth and metastasis, chemosensitivity and apoptosis (Fig. 3B).

**ANP Affects the NHE-1-Regulated pH in Cancer Cells Through a Frizzled Receptor-Mediated Mechanism**

One of the best described pH regulators, concurring to minimize changes in pHᵢ and to maintain tumor cell viability, is the amiloride-sensitive Na⁺/H⁺ exchanger isoform 1 (NHE-1), a plasma membrane protein that exchanges Na⁺ and H⁺ ions according to their concentration gradients [53]. It has been demonstrated that cytoplasmic alkalinization, that is an early event in tumorigenic transformation, is driven by a stimulation of NHE-1, which seems to represent the only system able to regulate pH homeostasis in cancer extracellular microenvironment, more acidic than normal tissues [54].

More than fifteen years ago, it has been reported that ANP is able to modify the intracellular pH by inhibiting or stimulating the NHE-1, depending on the cell type [55, 56]. In the 2005, Baldini and collaborators demonstrated that in HepG2 hepatoblastoma cells, ANP induced intracellular acidification by decreasing NHE-1 expression and
Fig. (2). Schematic representation of the Wnt/β-catenin signaling activation (top) and β-catenin and E-cadherin distribution in normal and transformed cells (bottom). In normal epithelial cells such as colonocytes, Wnt pathway is not activated and β-catenin (β-cat) and E-cadherin (E-cad) are mainly located at cell-cell junction; activation of Wnt signaling in tumor cells, and specifically in colon carcinoma cells, leads to β-catenin translocation into the nucleus and loss of E-cadherin at the cell contacts. **Bottom panels:** confocal microscopy images from our laboratory showing the different intracellular distribution of β-catenin and E-cadherin (green hue) in colonocytes and in colon adenocarcinoma cells (DHD/K12/Trb cell line). **Red hue:** nuclei were stained with propidium iodide. **APC, adenomatous polyposis coli; AKT, Serine/Threonine Protein Kinase; β-cat, β-catenin; CBP, CREB-binding protein; CK, casein kinase; DKK, Dickkopf; DSH, Dishevelled; GBP, GSK3-binding protein; GSK, glycogen synthase kinase; LRP, LDL receptor-related protein; P, phosphorylation; sFRP, secreted Frizzled-related protein; TCF, T-cell factor.**

activity [57]. These Authors suggested that the ANP effect on pH was mediated by NPR-C receptors. Owing the crucial role of NHE-1 in maintaining the pH homeostasis and preserving survival of cancer cells (Fig. 3A), it could be possible that a NHE-1-dependent intracellular acidification induced by ANP in tumor cells might also mediate its anti-proliferative activity by acting on the Wnt signaling. Starting from this hypothesis, we have recently demonstrated [30] that ANP triggers a NHE-1-mediated increase of the intracellular acidity in colorectal cancer cells and inhibits the Wnt/β-catenin signaling simultaneously. Conversely, Wnt1a, a Wnt signaling specific activator, affects the intracellular pH in an opposite fashion, inducing a prompt and dramatic intracellular alkalinization. The ANP-induced effect on pH is prevented by pre-treatment with the Frizzled ligand Wnt1a, used to saturate the specific receptor. This indicates that the NHE-1-mediated increase of the intracellular acidity triggered by the natriuretic hormone is possibly mediated by a mechanism relying on a Frizzled receptor/ANP direct interaction [30]. In that paper, we also reported that ANP induces a prompt decrease of pAkt$^{T308}$ - the active form of this enzyme - resulting in a reduction of the Akt-mediated inactivation of GSK-3β via Ser$^9$ phosphorylation. The dual function of Akt in both inactivating phosphorylation of GSK-3β at Ser$^9$ [36] and activating phosphorylation of NHE-1 at Ser$^{648}$ [58] suggests that Akt reduced activity could be a common molecular event underlying the ANP-induced inhibition of both Wnt/β-catenin signaling cascade and NHE-1 activity. Our data are partially confirmed by recent results obtained by Vesely and collaborators [59], demonstrating that ANP functions as inhibitor of AKT in human colorectal, pancreatic, and renal cancer cells, as part of its anticancer mechanism(s) of action.

Our recent results, provide evidence for an additional mechanism of ANP action that involves a cross-talk between the Wnt signaling cascade and the NHE-1-regulated intracellular pH in tumor cells (Fig. 4). In neoplastic cells, Wnt signaling causes a DSH-mediated phopho-activation of Akt that concomitantly might inactivate GSK-3β by phosphorylation at Ser$^9$ and activate NHE-1 by phosphorylation at Ser$^{648}$, leading to β-catenin stabilization and intracellular alkalinization, respectively. Both phenomena result in increased tumor cell proliferation, invasion, and metastatization, as well as in decreased chemosensitivity. ANP, possibly acting as a Frizzled ligand that antagonizes the Wnt signaling, reduces the DSH-mediated Akt activity. This causes two concomitant effects: i) the recovery of the GSK-3β activity at the “distruption complex,” that leads to increase of β-catenin degradation, APC-mediated β-catenin nuclear export and
ANP Affects Wnt/β-catenin Pathway and NHE-1 Activity in Cancer

**Fig. (3).** A. Generation of extracellular acidosis during tumor growth and strategy adopted by cancer cells to survive to extracellular acidic stress. Tumor cells are characterized by an alkaline cytosolic pH and an acidic extracellular pH: this pH gradient, whose preservation is crucial for tumor cell survival, is maintained by cancer cells through various pH regulators over-expressed on tumor cell surface, including Proton Pumps (PP) and Sodium/Proton Exchangers (NHE). B. Schematic representation of the effect of PP and NHE inhibitors on tumor microenvironment and cancer cell proliferation and survival; PP or NHE inhibitors, by decreasing the activity of the specific pH regulators, induce intracellular acidification that ultimately results in inhibition of tumor growth and metastasis, chemosensitivity and apoptosis.

**CONCLUSION AND FUTURE PERSPECTIVES**

The antitumor activity of ANP has been mainly related to its interaction with the specific receptors NPRs [3-9, 20], through which this natriuretic hormone inhibits some metabolic targets crucial for cancer development, including the GTPase RAS [21, 60, 61], the MEK1/2 kinases [22, 62] and ERK 1/2 kinases [23] (Fig. 5 and Table 1). Mitogen stimulation of RAS and ERK 1/2 as well as the VEGF receptor signaling are also inhibited by ANP [21, 24, 61, 63]. Signal activators of genes leading to malignancy, such as STAT3, and crucial molecules associated with cancer development, such as c-Jun-N-terminal kinase-2 and c-Myc, are also inhibited by ANP [25, 30, 64].

In addition to its ability to affect the Ras-MEK1/2-ERK1/2 kinase cascade, functioning as a multikinase inhibitor, we have recently demonstrated, for the first time, that Wnt signaling and the pH regulator NHE-1 are innovative...
Fig. (4). Proposed model for the mechanism of ANP action involving a cross-talk between the NHE-1-regulated intracellular pH and the Wnt signaling. APC, adenomatous polyposis coli; AKT, Serine/Threonine Protein Kinase; β-cat, β-catenin; CBP, CREB-binding protein; CK, casein kinase; DKK, Dickkopf; DSH, Dishevelled; GBP, GSK3-binding protein; GSK, glycogen synthase kinase; LRP, LDL receptor-related protein; NHE, Sodium/Proton Exchanger; P, phosphorylation; TCF, T-cell factor. [Adapted from 30].

Fig. (5). Receptors and cellular signaling pathways implicated in the antitumor effect of ANP. NHE-1, Na⁺/H⁺ exchanger isoform 1; NPRs, Natriuretic peptide receptors; TKR, Tyrosine kinase receptor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor. Details and main function/s of the different molecular components, and references supporting the role of each molecular component and pathway in ANP antitumor action are reported in (Table 1).
targets for anticancer activity of ANP [30] (Fig. 5 and Table 1). In particular, the natriuretic hormone exerts its inhibitory effect on cancer cell proliferation by concomitantly influencing the Wnt/β-catenin pathway and NHE-1-regulated intracellular pH. These two effects seem to be triggered through a Frizzled receptor-mediated mechanism and firstly driven by a reduced Akt activity [30, 59]. Therefore, it acts as Frizzled ligand antagonizing the Wnt signaling and as NHE-1 inhibitor. In the last years growing evidence sustains the roles of both acidic tumor microenvironment and Wnt/β-catenin pathway activation in cancer initiation and progression and several molecular components in the Wnt signaling as well as the pH regulators have been proposed as novel targets for cancer prevention and therapy. In this context, our innovative results reinforce the validity of ANP as antitumor drug that, for the peculiarity of simultaneously targeting two processes crucial for neoplastic transformation and solid tumor survival, might be considered as a “magic bullet” against cancer, useful for the development of both preventive and therapeutic strategies.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

We would like to thank Federica Andreola, Luana Mercuri, Noemi Moroni, Rossana Psaila, Francesca Wannenes and Manuela Zonfrillo, for their valuable contribution to the researches carried out in our laboratory on ANP antitumor activity and mechanism(s) of action, and to the ongoing study on the role of WNT/β-catenin signaling in cancer. We are also grateful to Dr. Giuseppe Nicotera for his scientific secretariat support. This work was partially supported by National Research Council of Italy and partially by MIUR (CNR Research Project on Aging, Grant n, B81J12001510001 to PP).

ABBREVIATIONS

ANP = Atrial Natriuretic Peptide
APC = Adenomatous Polyposis Coli
AKT = Serine/Threonine Protein Kinase
β-cat = β-catenin
CBP = CREB-binding protein
CK = Casein Kinase
DKK = Dickkopf
DSH = Dishevelled
EGF = Epidermal Growth Factor
EGFR = Epidermal Growth Factor Receptor
ERK = Extracellular Signal-Regulated Kinase
Fz = Frizzled
GBP = GSK3-Binding Protein
GSK-3β = Glycogen Synthase Kinase
IGFR = Insulin Growth Factor Receptor
KP = Kaliuretic Peptide
LANP = Long-Acting Natriuretic Peptide
LDLR = LDL Receptor Repeats
LEF = Lymphoid enhancer-binding factor
LRP = LDL Receptor-Related Protein
MEK = Mitogen-Activated Protein Kinase
NHE-1 = Sodium/Proton Exchanger Isoform 1
NPRs = Natriuretic Peptide Receptors
PP = Proton Pump
RAS = Rat Sarcoma
TDK = Dickkopf
VEGF = Vascular Endothelial Growth Factor
VEGFR = VEGF Receptor
VSDL = Vessel Dilator

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