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Biomedical Journal

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Review Article

Modified natriuretic peptides and their potential roles in cancer treatment



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ARTICLE INFO

Article history:

Received 23 February 2021

Accepted 28 June 2021

Available online 6 July 2021

Keywords:

Natriuretic peptides (NPs)

Cancer treatment

Natriuretic peptide modification

Peptide therapy

ABSTRACT

The natriuretic peptide family (NPs) is a group of natural endocrine hormones, containing a 17-amino acid ring structure connected by disulfide bonds of two cysteines. In this review, the members of the natriuretic peptide family and their corresponding receptors as well as the anti-cancer effects are introduced. Four cardiac hormones of NPs (ANP, VD, KP and LANP) can effectively inhibit the growth of human small cell lung cancer, breast cancer and other tumors and significantly reduce tumor volume *in vivo*. The *in vitro* experiments also show that cardiac hormones, CNP and urodilatin can effectively inhibit the growth of most tumor cells. We then further summarized the anti-cancer mechanism of natriuretic peptides. Finally, we introduce several methods that modify natriuretic peptides, leading to enhance their stability and prolong the biological effects of these peptides, which might be helpful for the clinical application in the future. Peptide therapy is a very promising field for cancer treatments since they can induce the death of cancer cells without dramatically affecting normal cells. The synthesis of a useful and stable natriuretic peptide can enhance the effect of cancer treatments and significantly reduce drug resistance and toxicity.

Cancer is a very complex disease, which manifests as abnormal cell proliferation and, at the same time, invades normal tissues and metastasizes to distant organs [1]. With the increasing incidence and deaths, cancer has become the

leading cause of death and a significant public health problem all over the world [2]. Although current treatments have improved the overall survival rate, the traditional treatment (e.g., surgery, radiotherapy, and chemotherapy) and targeted

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Peer review under responsibility of Chang Gung University.

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<https://doi.org/10.1016/j.bj.2021.06.007>

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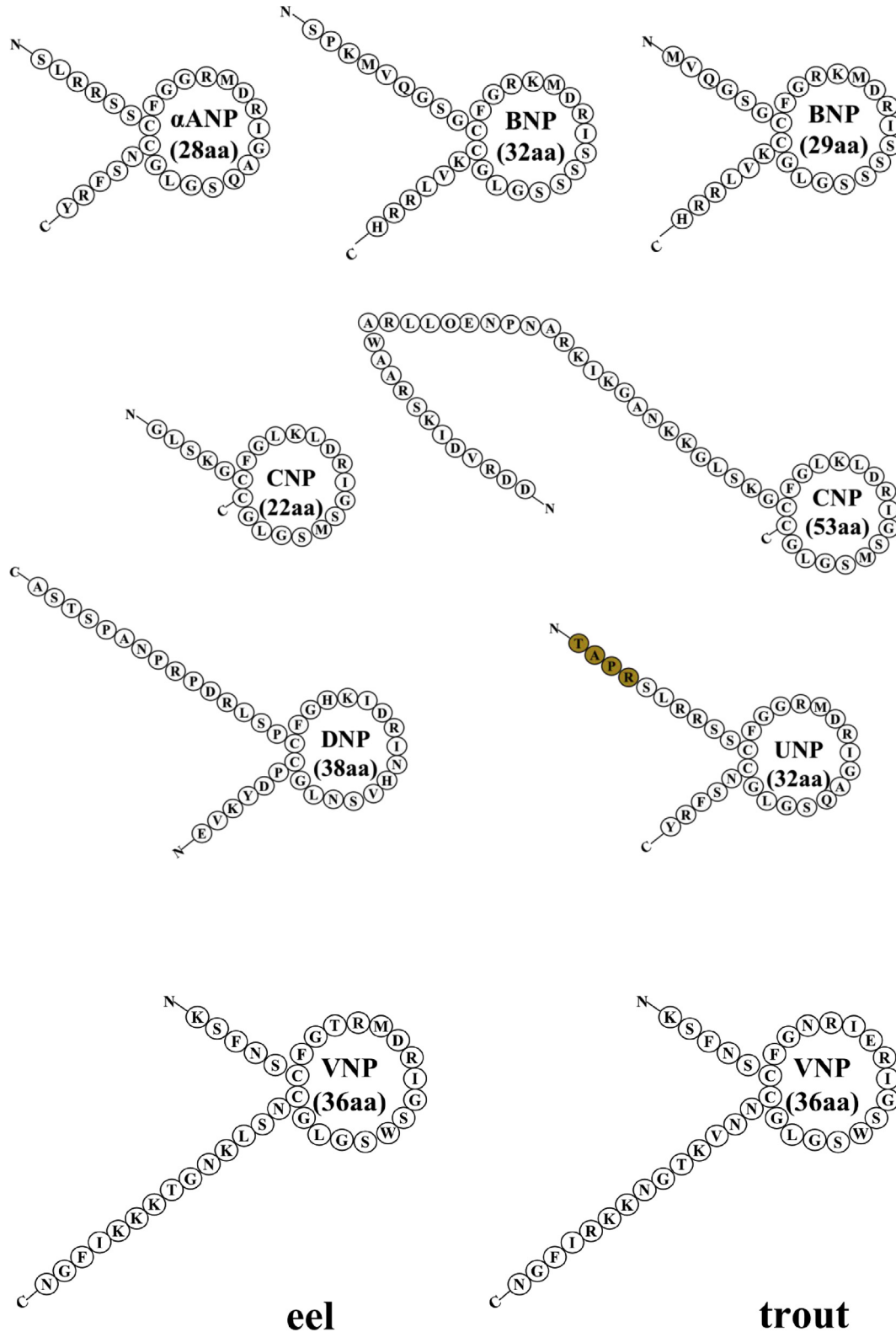


Fig. 1 Schematic structures of the NPs. Amino acid sequences of α ANP, BNP, CNP, DNP, UNP and ventricular natriuretic peptides (VNP) with biological activity in vertebrates, the amino acid in urodilatin are identical to the four C-terminal a.a. of kaliuretic peptide and identical (Yellow mark). ANP, BNP, CNP, DNP and UNP have been identified in mammals, while VNP has been identified in tetrapods and teleostean fish.

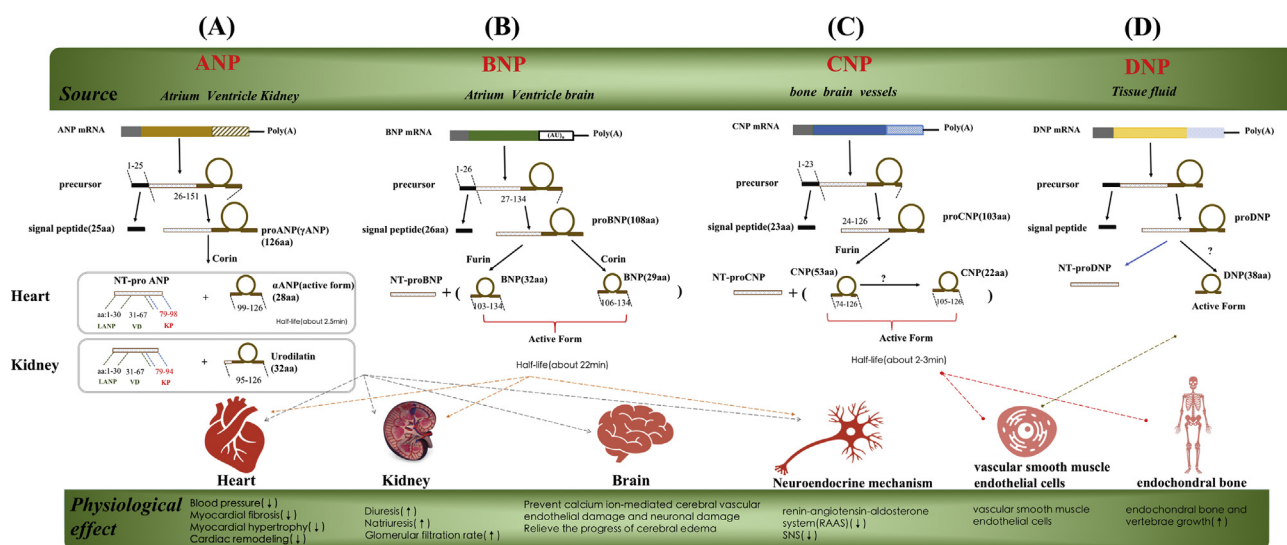


Fig. 2 Human ANP(A), BNP(B), CNP(C), and DNP(D) gene structure, biosynthetic pathway, source and primary function. (A) ANP is originally synthesized as a 151-amino acid precursor, which is further synthesized as a 126-amino acid pro ANP (γ -ANP) in the atrial myocytes of the heart, along with a 25-amino acid signal peptide. After removal of signal peptide by signal peptidase in the endoplasmic reticulum, pro ANP is stored in atrial cardiomyocyte granules. In the heart, then pro ANP is proteolyzed into an active α ANP (28-amino acid) and an inactive NT-proANP by the convertase Corin. The NT-proANP contains other three natriuretic peptides, the long-acting natriuretic peptide (LANP), the vessel dilator (VSDL), and the kaliuretic peptide (KP); In the kidney, the ANP prohormone is cleaved between amino acid (a.a.) 94 and 95; (B) Ventricular myocytes produce a precursor of 134 amino acid (pre-pro BNP), which is further cleaved to obtain a signal peptide and pro-BNP (108-amino acid). During the secretion process, pro-BNP is cleaved by enzyme Furin into a biologically active BNP (32-amino acid), and another fragment of biologically inactive NT-proBNP (76-amino acid) which comprises an N-terminal fragment. The enzyme Corin can also proteolyze it into an active BNP (29-amino acid). Both are secreted from the ventricle into the peripheral circulation; (C) When CNP is released from cells it is cleaved into a 53 amino acid peptide with biological activity and inactive 50 amino acid N-terminal fragment, NT-proCNP by Furin. CNP-53 is further cleaved to yield a 22 amino acid peptide that is almost identical biological activity *in vitro* as CNP-53. The precise mechanism, regulating the formation of CNP-22 remains unknown; (D) Pro-DNP is cleaved into 38-amino acid with biological activity (the mechanism regulating remains unknown).

molecular therapy still have numerous shortcomings [3]. Therefore, there is an urgent need for developing new anticancer drugs, especially those that can kill cancer cells while minimizing drug resistance and side effects. Recently, it has been reported that anticancer peptides might become a promising candidate for cancer treatment [4]. Natriuretic peptide is one of such kind of peptides generated in living organisms. It is a promising drug discovery model although most of the work still stayed at the research level. Most likely, the short half-life of the peptides and their large molecules hindered the further application.

Therefore, we review the current research progress of natriuretic peptides to illuminate the possible ways to solve these problems. On the basis of current research, transforming and modifying natural peptides is a feasible way to remedy the shortage of insufficient half-life and enhance the physiological effects *in vivo*. Two possible methods might be used to reach this goal. Firstly, the well-studied virus packaging format was applied to construct recombinant natriuretic peptide genes. Secondly, natriuretic peptide hormone is used in conjunction with traditional anticancer drugs simultaneously to increase the anti-cancer effect and decrease the side effects of traditional rivals [5]. Overall, these methods

could provide directions for future research on new peptide drugs, which might be a hot spot of future studies.

The source, biosynthesis and physiological functions of natriuretic peptide family

Natriuretic peptide family (NPs) mainly consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroaspis natriuretic peptide (DNP), urodilatin (UNP), and ventricular NP (VNP) [Fig. 1].

Cardiac NPs including ANP, BNP, DNP and VNP are endocrine hormones secreted from the heart, which have a C-terminal 'tail' sequence of variable length that extends from the intramolecular ring structure formed by a disulfide bond [6] [Fig. 1]. UNP is secreted from the kidney, while CNP is principally a paracrine factor in the brain and periphery [7]. CNP lacks the 'tail' sequence, functions as a paracrine or autocrine factor in the brain and peripheral tissues such as the endothelium, suggesting that it is the ancestral type of the NP family while other NPs appeared later in the vertebrate phylogeny [8][Fig. 1]. All the structures of active natriuretic peptides with a ringed structure formed by intramolecular

cysteine disulfide linkages, and this circular structure is the necessary spatial structure for the binding of active NPs to their receptors [9].

Atrial natriuretic peptide (ANP) and urodilatin (UNP)

The atrial natriuretic peptide (ANP) is a small molecule peptide hormone composed of 28 amino acid residues [Fig. 1], and derived hormone were identified in 1984 [10]. Later, several other groups were isolated and purified and named as atrial natriuretic factor (ANF) or ANP [11]. The human ANP gene is located on the short arm of chromosome 1 and ANP mRNA is expressed in the brain, pituitary, kidney but it is most abundant in the heart, especially in the atrium [11]. ANP is proteolytically cleaved at the cell membrane by the convertase Corin. Then it is synthesized as a 98-amino acid inactive precursor (pro-ANP), that contains other three natriuretic peptides, the long-acting natriuretic peptide (LANP) a.a. 31–67 (i.e. vessel dilator), a.a. 79–98 (kaliuretic peptide) and a.a. 99–126, the vessel dilator (VD), and the kaliuretic peptide (KP). Meanwhile, the pro-hormone that gives rise to the mature ANP (COOH-terminal) [12] [Fig. 2A]. ANP will circulate in the bloodstream and act on various organs. In humans, after i.v. injection, the half-life of ANP is 2–4 min: it can be degraded by metallo-endopeptidases as well as by binding to its clearance receptor, i.e., NPR-C [13]. The elimination organs include the liver, kidney, lung, skeletal muscle, and blood vessels. Moreover, ANP can improve the hemodynamic effect, lower blood pressure, resist the renin-angiotensin-aldosterone system (RAAS), dilate blood vessels [13][Fig. 2A].

Urodilatin is a peptide hormone formed by a differential processing of the ANP prohormone in the kidney, where instead of cleaving the 126 amino acid (a.a.) prohormone between a.a. 98 and 99 to form ANP and kaliuretic peptide it cleaves this prohormone between a.a. 95 and 96 [14][Fig. 2A]. UNP originally extracted from human urine that corresponds to the family of natriuretic-vasorelaxant peptides found earlier in heart atria. In the kidney, the cleavage of the ANP prohormone causes four amino acid from the C-terminal end of kaliuretic peptide (i.e. threonine·alanine·proline·arginine) being attached to the N-terminus of ANP [15][Fig. 1].

Brain natriuretic peptide (BNP)

BNP and ANP are also called cardiac hormones, mainly produced by atrial and ventricular myocytes. BNP was originally isolated from porcine brain tissue which is also called as brain natriuretic peptide [16]. Subsequent studies found that BNP can also be isolated from the heart, and the amount of BNP secreted by the heart is more than that of the brain [17] [Fig. 2B]. The human BNP gene is located on chromosome 1 and its transcript is translated to a 134-amino acid peptide. The 26-amino acid signal peptide is then removed, proBNP1-108 is converted to a biologically active peptide BNP1-32 and an inactive NT-proBNP1-76 [18]. The normal heart releases a proteolytically processed BNP1-32 and NT-proBNP, while the diseased heart secretes high amounts of unprocessed/glycosylated proBNP or inappropriately processed BNPs. But circulating proBNP has recently been identified in normal subjects. Therefore, proBNP has a longer half-life than BNP32,

circulating concentrations of proBNP are known to increase in certain pathological states [30]. The enzyme Corin can also proteolyze proBNP into an active BNP (29-amino acid) [18] [Fig. 2B]. The half-life of BNP29 in the plasma is 12–23 min, and its expression is controlled by adaptive mechanisms in response to myocardial stress [19]. NT-proBNP has a longer half-life and thus has a higher plasma concentration than BNP [20].

Nesiritide is the recombinant form of human BNP and is the only member of the natriuretic peptide family approved by the FDA for the treatment of decompensated heart failure worldwide [21]. Its physiological functions mainly include natriuretic diuretic, vasodilating, inhibition of renin-angiotensin-aldosterone system (RAAS), anti-proliferation [20] [Fig. 2B]. Moreover, in the cancer research, the study suggested that BNP could also be used as a biomarker for cardiac damage of breast cancer patients after radiotherapy [22]. It also been reported that plasma BNP levels are elevated in patients with solid cancer and suggested that these increases are due to the cardiac response to cancer-related inflammation [23].

C-type natriuretic peptide (CNP)

CNP was first isolated from porcine brain, and CNP is paracrine messenger, notably in bone, brain, and vessels [24]. However in terms of tissue expression, CNP content majorly reside in brain, reproductive tissue and bone [25]. CNP also has a ring structure and is highly homologous to both ANP and BNP which lacks the carboxy-terminal extension [26]. CNP can be expressed broadly in the central nervous system and peripheral tissues. The Human CNP gene is located on chromosome 2 and it consists of 2 exons and its transcript is translated to a 126-amino acid peptide (pre-pro CNP), which after cleavage of first 23 amino acid gets converted into pro-CNP, which is further cleaved into 53-amino acid with biological activity and an inactive NT-proCNP by the enzyme Furin [27]; it is further processed into a 22-amino acid peptide (CNP-22) with biological activity, but the precise mechanism regulating the formation of CNP-22 remains unknown [Fig. 2C]. The primary biologically active forms are CNP-22 and CNP-53 [Fig. 1]. The CNP is a highly conserved natriuretic peptide, the amino acid sequences of CNP-22 are the same in all mammals, but only three amino acid substitutions in the CNP-53 sequence have been observed so far [28]. Compared to ANP and BNP, the plasma half-life of CNP is much shorter and is about 2–3 min in humans [29]. The physiological activity of CNP is different from that of other members and it has been reported that endothelial CNP regulates distal arteriolar and capillary blood flow, and inhibits vascular smooth muscle cell proliferation [30][Fig. 2C]. Moreover the study showed that CNP is a potent stimulator of endochondral bone and vertebrae growth, which is also a potent stimulator in the craniofacial region, crucial for midfacial skeletogenesis [31].

Dendroaspis natriuretic peptide (DNP)

Dendroaspis natriuretic peptide (DNP), a 38-amino acid peptide containing a 17-amino acid disulfide ring structure [Fig. 1], was isolated from the venom of *Dendroaspis angusticeps* snake

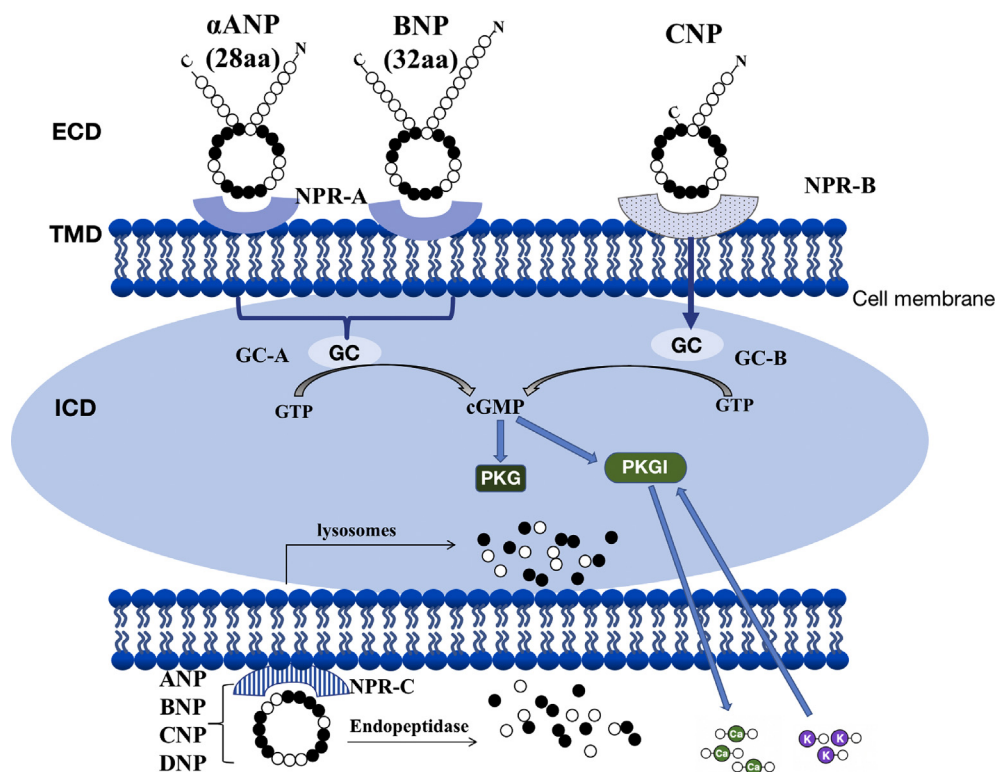


Fig. 3 Natriuretic peptides and their receptor binding and themolecular signaling pathways. When the receptor binds to the ligand, it triggers the production of catalytic guanylate cyclase (cGMP), increases the level of cGMP in the cell, activates downstream signaling pathways, and causes physiological effects. (A) ANP and BNP have high specificity and affinity for binding to NPR-A (Binding association with NPR-A: ANP > BNP > CNP > DNP); (B) CNP without C-terminal has high activity for binding to NPR-B(Binding relationship with NPR-B: CNP > ANP > BNP > DNP); (C) ANP/BNP/CNP/DNP all play their biological roles by mediating the intracellular cGMP level; (D) NPR-C is a scavenging receptor that can scavenge NPs and reduce their concentration, which is comparable to the affinity of the three NPs. (Binding relationship with NPR-C: ANP ≥ CNP ≥ BNP ≥ DNP).

by Schweitz [32]. Compared with others, DNP has a long C-terminal with 15 amino acid [Fig. 1], which makes DNP more resistant to Neutral endopeptidase's degradation (NEP) than other natriuretic peptides. DNP-like immunoreactivity has been detected in human atria, canine myocardium, vascular smooth muscles and plasma [33]. It is elevated in the plasma of humans with congestive heart failure [33]. Although the main synthetic site of DNP and its specific binding sites are yet to be defined [Fig. 2D]. DNP has been shown to stimulate GC activity in vascular smooth muscle and endothelial cells [34] [Fig. 2D].

Ventricular NP (VNP)

Ventricular NP (VNP) is a novel type which has been isolated from the cardiac ventricle of eel and trout [35][Fig. 1]. Eel VNP has a uniquely long C-terminal 'tail' sequence with 14 amino acid residues [36], which is longer than those of ANP, UNP (five residues), BNP, DNP (six residues) and CNP (no residue). Heterologous fish VNP exhibited more potent vasorelaxant

activity than homologous mammalian ANP in the isolated coronary artery of dogs [8]. VNP has not been molecularly identified in mammals but its presence is suggested from physiological studies [8].

Types, tissue distribution and binding characteristics of NPRs (natriuretic peptide receptors)

NPs exhibit their biological activity by binding to their receptors with high affinity. The three distinct cell surface receptors called natriuretic peptide receptors A, B and C (NPR-A, NPR-B and NPR-C). NPR-A and NPR-B are particulate guanylyl cyclase (GC)-linked receptors that mediate increases in cGMP upon activation [37]. Among the NP components, NPR-C has been described, at the time of its discovery, as the clearance receptor of NP devoid of any physiological functions. Emerging roles of NPR-C, however, have been highlighted over the last few years in relation to its effects on the

cardiovascular system and other organs. These effects appear to be directly mediated through distinct cAMP-dependent intracellular mechanisms [38]. In addition to the above three types of receptors, Kashiwagi et al. [39] also found a new kind of NPR in eel in 1995, named D-type natriuretic peptide receptor (NPR-D), which is a homology of NPR-C, and the cDNA sequence homology of both in eel is up to 70%.

NPR-A is mainly expressed in the great vessels, adrenal glands, and kidneys [40], while the expression of NPR-B is reported in various organs such as bone, brain, heart, kidney, liver and in some cancers [41]. The expression of NPR-C is also ubiquitous and is known to be expressed in cardiomyocyte, fibroblasts, vascular smooth muscle cells, heart, adrenal glands, kidney and in some cancers [42]. However, NPR-D has been identified in the eel which shows a high-affinity binding between eels and three NPs in rats [43]. NPR-D is abundant in the brain and a member of the receptor subfamily with a short cytoplasmic C-terminal tail [44].

Moreover, in mammals, the three NPRs have different affinity for different NPs [45]. NPR-A selectively binds ANP, BNP, CNP [Fig. 3], DNP, and UNP [Fig. S1], while it has a higher affinity to ANP. In general, the affinity of NPs is ANP > BNP > CNP > DNP ≥ UNP [46]. Whereas NPR-B is more likely to bind CNP. Finally, NPR-C has low ligand requirements and can also bind ANP, BNP, CNP, and other polypeptides with a high homologous structure affinity. Furthermore, the affinity between NPs and NPR-C receptor is ANP ≥ CNP ≥ BNP ≥ DNP [47][Fig. 3].

Structure and function of NPR-A, NPR-B

The structure of NPR-A is similar to NPR-B. Both of them have extracellular domain (ECD), transmembrane domain (TMD), and intracellular domain (ICD) [48][Fig. 3]. As mentioned above, the binding affinity of ANP to NPR-A is 10 times higher than that of BNP, and CNP can only bind to NPR-B, which may be related to its special structures. Further, DNP and urodilatin can also play their physiological roles when combined with NPR-A [49]. They ultimately activate guanylate cyclase and increase the intracellular cGMP content [Fig. S1]. ANP increases the cGMP in the cytoplasm by combining with NPR-A. After that, it activates cGMP-dependent protein kinase G (PKG), up-regulates the expression level of genes related to transcription and translation, and affects cell growth, proliferation, apoptosis, immune, and other physiological functions [Fig. 3]. Compared with normal human vascular endothelial cells, NPR-A is highly expressed in lung cancer, skin cancer, ovarian cancer, prostate cancer cell lines [50], etc. Meanwhile, the lack of NPR-A can prevent experimental animals from developing tumors. These results indicate that both ANP and NPR-A play critical biological functions in tumor occurrence and development. Research illustrates that DNP binds to NPR-A, it also activates cGMP and becomes the second messenger to activate PKG, which enhances Ca²⁺-activated K⁺ channel [IK(Ca)] and transient outward potassium channel [SCOTc] to cause smooth muscle relaxation [51]. Additionally, GTP can activate Phosphatase C (PLC) to decompose phosphati-

dylinositol biphosphate (PIP2) on the membrane into two intracellular second messengers diacylglycerol (DAG) and inositol triphosphate (IP3) [51]. IP3 binds to the receptor and thereby activates protein kinase C (PKC), causing the IR calcium store to release Ca²⁺. When the calcium store is depleted, the store-operated calcium channel (SOCC) on the cell membrane will be activated, which promotes extracellular calcium influx, and enhances [IK(Ca)], causing smooth muscle relaxation [51][Fig. S1]. UNP binds to the NPR-A, -B and -C receptors with binding curves superimposable with ANP [51][Fig. S1]. Thus, based upon this knowledge, our hypothesis was that urodilatin would have similar anti-cancer effects to ANP [52].

Structure and function of NPR-C

NPR-C also contains ECD, TMD, and ICD consisting of only 37 amino acid residues, but the ICD has no guanidine cyclase activity. NPR-C clears natriuretic peptides from the circulation through receptor-mediated internalization and degradation and it is released by endothelial cells [53]. Although it can be stimulated by NPs and bind with ANP, BNP, CNP, and DNP with different affinity, it cannot activate the cGMP pathway, only through internalization and enzymatic degradation (the ligand-NPR-C complex is subjected to internalization and degradation [54]), and then NPR-C returns to the cell surface. NPs can also be removed by neutral endopeptidase (NEP) [55], which is present in the blood vessels and kidneys. It is worth mentioned that DNP is more resistant to the degradation of NEP due to its unique long amino-terminal of 15 amino acid residues [Fig. S1].

Biological metabolic pathways of natriuretic peptides

There are three circulating pathways for the removal of natriuretic peptides *in vivo*. One is cleaved by a neutral endopeptidase that is mainly present in the lung, the brush border of the proximal renal tubules, and the surface of endothelial cells; Secondly, it is mediated by the NPR-C to engulf the natriuretic peptides and degrade them by the lysosome [47][Fig. 3]. These two pathways are the most important ways to eliminate natriuretic peptides. Besides, a small number of natriuretic peptides can be directly filtered through the glomerulus.

Therapeutic application of natriuretic peptides in cancer

Therapeutic application of NPs *in vivo*

Natriuretic peptides can dramatically stop the growth of human pancreatic adenocarcinomas in athymic mice and decrease their tumor volume [12]. Vesely D.L. found that these cardiac hormones are given subcutaneously for 1 month, up to 80% of the human pancreatic adenocarcinomas [12] and 86% of human small-cell lung carcinomas [56] growing in athymic

Table 1 Anti-cancer effect and mechanism of natriuretic peptide.

NPs Types	Tumor cells types	Tumor-suppressor mechanism	References
Cardiac hormones (ANP, KP,VD, LANP)	Human prostate cancer cells	①Ras-MEK1/2-ERK1/2 signal transduction pathway (↓) ②VEGF/VEGFR2 signaling pathway (↓) ③the expression of c-fos and c-jun proto-oncogenes (↓)	[66–69] [79] [86]
	Human colorectal adenocarcinoma	Wnt/β-catenin signaling pathway (↓)	[60,65,76]
	Human kidney cancer cells	Regulate the balance of the pH value of the tumor extracellular microenvironment	[89]
	Human pancreatic cancer cells	①Wnt/β-catenin signaling pathway (WNT-3a, sFRP-3↓) ②VEGF/VEGFR2 signaling pathway (↓) ③STAT3 activation (↓) ④Regulate the balance of the pH value of the tumor extracellular microenvironment	[60,65,76] [79] [84] [89]
	Human non-small cell lung cancer cells	①VEGF/VEGFR2 signaling pathway (↓) ②STAT3 activation (↓)	[79] [83]
	Human hepatocellular cancer cells	the expression of c-fos proto-oncogenes (↓)	[85]
Cardiac hormones urodilatin	Human renal cancer cells	①Wnt/β-catenin signaling pathway (↓) ②the expression of c-fos proto-oncogenes (↓)	[60,65,76] [85]
Cardiac hormones (ANP, KP,VD, LANP)	Human small cell lung cancer cells	the expression of c-fos and c-jun proto-oncogenes (↓)	[85]
CNP			
ANP	Human colon cancer cells	Regulate the balance of the pH value of the tumor extracellular microenvironment	[89]
Cardiac hormones	Human breast adenocarcinoma cells	inhibition of DNA synthesis	[90,91]
VD	Human angiosarcoma cells		
	Human squamous lung cancer cells		[92]
CNP (combine with sildenafil)	RMS cells	Raf/MEK/ERK pathway	[99]

mice can be dramatically eliminated. Similarly, human breast cancers transplanted in athymic mice can be eliminated without surgery with these cardiac hormones [57].

Three of four cardiac hormones (ANP, VD, KP) synthesized by the ANP gene can eliminate human squamous cell lung carcinomas in athymic mice [58].

Effects of NPs on cancer cell lines in vitro

The four cardiac hormones, namely ANP, VD, KP and LANP, have very potent effects of eliminating up to 97% of prostate cancer cells within 24 h of treatment [72]. Natriuretic peptides can inhibit the progression of pancreatic-, breast-, small cell lung-, and prostate cancer *in vitro* and have been proposed as promising treatments for cancers [59] since they induce the death of cancer cells without affecting normal cells [60].

It has been reported that three peptide hormones (KP, VD, LANP) had significant effects on reducing the number of human renal carcinoma cells [61]. The study indicated that when the concentration of KP, ANP and LANP were increased to 100 μM, the number of renal cancer cells significantly decreased by 70–74% within 24 h [61]. Urodilatin and the four cardiac hormones have potent anti-cancer effects by eliminating up to 81% of renal carcinoma cells within 24 h of treatment [62]. ANP, LANP, KP and CNP can also inhibit the growth of human small-cell lung carcinoma cells [63]. Each of

these peptides, except BNP, have anticancer effects *in vitro* when given in concentrations above those normally circulating in the human body, i.e. pharmacological concentrations [4].

Anticancer effects of natriuretic peptides: experimental evidence

Inhibition of the Ras-MEK1/2-ERK1/2 signal transduction pathway

Rat Sarcoma Bound Guanosine Triphosphate (Ras) is a type of monomeric protein that can bind to guanosine triphosphate (GTP) and participate in intracellular signal transduction. It will be in an activated state when Ras protein is combined with GTP (GTP-Ras). On the contrary, it will be in a deactivation state combined with GDP (GDP-Ras). Under normal circumstances, Ras is essentially inactivated. Usually, Ras protein is inactivated state. Ras gene family includes H-Ras, K-Ras and N-Ras. The most common mutation is a point mutation, about 25%–30% of human malignancies [64] have point mutations in the Ras gene and activated Ras High protein expression.

Epidermal Growth Factor (EGF) promotes tumor growth. The overexpression of its receptor (EGFR) is seen in various

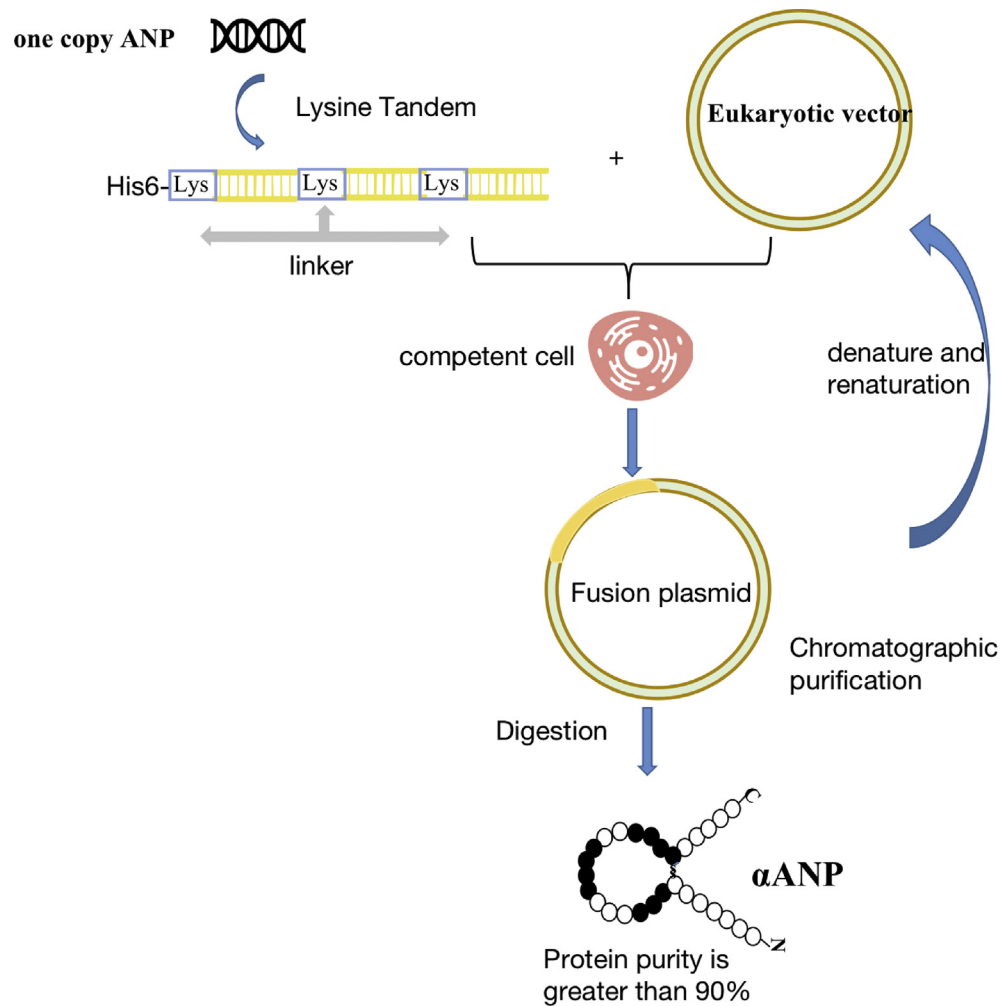


Fig. 4 Preparation of ANP by expressing multiple copies of genes in tandem. Three ANP genes were connected in tandem, and the fusion protein His6-K-ANP-K-ANP-K-ANP (His6-ANP3) was recombinantly expressed in *E. coli*. The linker (lysine) was inserted between ANP and the fusion tag and ANP. (Lys: lysine).

malignant tumors. Mitogen-activated protein kinase (MEK) is an intracellular serine/threonine protein kinase (AKT) activated by various extracellular and intracellular stimulus signals. Moreover, MAPK activated protein kinase (MEK) is the only kinase that can activate MAPK. Activation of the above factors and kinases will activate the intracellular Ras-MEK1/2-ERK1/2 signal transduction pathway, leading to the generation of malignant tumors, such as lung cancer, breast cancer, prostate cancer and pancreatic cancer [65].

SUN et al. [66] found that the cardiac hormone natriuretic peptides can inhibit Ras activation in human prostate cancer cells through direct action, and can also inhibit Ras activation through indirect action (inhibition of EGF to induce Ras protein). The direct inhibition is significantly stronger than the indirect inhibition. It was subsequently found that ANP and LANP with different concentration ranges inhibited the activation of ERK1/2 [67] while VD and KP inhibited MEK 1/2 activation in human prostate cancer cell lines [68]. Therefore, the main target of cardiac hormone natriuretic peptides is Ras-MEK1/2-ERK1/2 signal transduction pathway, and the

anticancer mechanism is mainly cGMP mediated inhibition of DNA synthesis of cancer cells [69][Table 1].

Inhibition of the Wnt/ β -catenin signaling pathway

β -Catenin is a multifunctional protein located at the intracellular side of the cytoplasmic membrane [70] that could cause the malignant growth of colon [71], renal [72] and pancreatic [73]. Secreted frizzled-related proteins (SFRPs) are extracellular inhibitors of Wnt signaling that act by binding directly to Wnt ligands or to Frizzled (Fz) receptors [74]. The Wnt signaling pathway is one of the critical signaling pathways involved in regulating embryo and organ development and tissue metabolism [75].

The cardiac hormone natriuretic peptides can directly inhibit the Wnt/ β -catenin signaling pathway by effectively reducing the level of β -catenin in human colorectal adenocarcinoma cells, human pancreatic cancer cells, and human renal cancer cells [60][Table 1]. Subsequently, natriuretic peptides were also discovered to be capable of directly or

indirectly affecting the Wnt signaling cascade and exerting their anti-proliferative activity in human pancreatic carcinoma cells and human colorectal adenocarcinoma cells [65]. Other studies showed that all the four cardiac hormones could also reduce the concentration of the WNT-3a in the Wnt pathway [65] and decrease the levels of the Secreted Frizzled-related protein-3 (sFRP-3) in pancreatic, colorectal and renal cancer cells [76].

Inhibition of the VEGF/VEGFR2 signaling pathway

Vascular endothelial growth factor (VEGF) is mainly involved in the proliferation, migration and survival of many cancer cells [77]. VEGF is a well-characterized mediator of tumor angiogenesis that functions primarily by binding and activating VEGF receptor 2 (VEGFR2) [78]. Experiments showed that cardiac hormones could significantly reduce the level of VEGF and VEGFR2 in human prostate cancer cells [79], and down-regulate the expression of VEGF and VEGFR2 in pancreatic adenocarcinoma cells and non-small cell lung cancer cells [79], thereby inhibiting the growth of cancer cells [Table 1].

Inhibition of the activation of STAT3

Stimulation of epidermal growth factor receptor (EGFR) by ligand(s) leads to activation of signaling molecules including STAT1 and STAT3, two members of the signal transducers and activators of transcription (STAT) protein family [80]. Signal transducers and activators of transcription (STAT) are cytoplasmic proteins with both signal transduction and transcription activation functions; their abnormal activation and overexpression can induce tumor formation [81]. Among the STAT protein family members, the overexpression of STAT3 is closely related to the biological behaviors and mechanisms of tumor proliferation, invasion, and metastasis [82]. Experiments revealed that natriuretic peptides play an anti-tumor effect by inhibiting the activation of STAT3 in human non small-cell lung cancers [83] and human pancreatic adenocarcinoma cells [84] [Table 1].

Inhibition of the expression of proto-oncogene products in the nucleus

Proto-oncogenes are “Housekeeping Genes” and are necessary to maintain the physiological activities of cells. c-Fos is a cellular proto-oncogene which dimerizes with c-Jun proto-oncogene to form AP-1 transcription factor, which upregulates transcription of genes involved in proliferation and cancer formation [85]. Experimental studies have found that cardiac hormones decreased c-Fos by 59%–61% in human hepatocellular cancer cells, by 74%–82% in small-cell lung cancer cells, and by 74%–82% in human renal adenocarcinoma cells. c-Jun was maximally reduced by 31%–61% in hepatocellular cancer cells, by 40%–65% in small-cell lung cancer cells, and by 43%–57% in renal cancer cells [85]. Moreover, cardiac hormones can inhibit the expression of proto-oncogene products in the nucleus by entering the core of prostate adenocarcinoma cells [86][Table 1], thereby inhibiting the proliferation and growth of cancer cells.

Increase acidity in cells

The survival of cancer cells and their adaptation to the extracellular acid environment are achieved by expressing many pH-regulating molecules on the cell surface, maintaining the balance of extracellular and intracellular pH. The Na⁽⁺⁾/H⁽⁺⁾ Exchanger Isoform 1 (NHE-1), as a critical pH regulator, can adjust the tumor cell microenvironment's pH, reduce the change of intracellular pH, and maintain tumor cells' viability [87]. While natriuretic peptides activate NHE-1 to increase cancer cells' intracellular acidity, Wnt/ β -catenin signaling is also inhibited. ANP can induce a decrease in the activity of pAktT308, and they act as Fz ligands to neutralize Wnt signals and cause the inactivation of GSK-3 β Ser9 phosphorylation when tumor cells are proliferating, invading, metastasizing, and chemically sensitive [88]. Cardiac hormones can be used as an inhibitor of Akt signaling in cancer cells such as colon cancer cells, pancreatic cancer cells, and kidney cancer cells [89][Table 1].

Inhibition of DNA synthesis

cGMP was found to be a key mediator of the inhibition of DNA synthesis. Cardiac hormones decreased DNA synthesis 69%–85% in human breast adenocarcinoma cells [90]. Four cardiovascular hormones decrease the number of angiosarcoma cells within 24 h via inhibition of DNA synthesis mediated in part by cyclic GMP [91]. Vessel dilator decreased DNA synthesis 85% in the squamous lung cancer cells, mediated in part by cyclic GMP, more than other peptide hormones [92] [Table 1].

Biosynthesis of natriuretic peptides

Tandem natriuretic peptides genes

In order to increase the yield, polypeptide genes can be connected in tandem and expressed by plasmids. The proteins can be processed and modified after translation in cells. Finally, fermentation products with high expression amounts will be cultivated as peptide drugs. Due to its small molecular weight, ANP is currently mainly prepared by recombinant expression to obtain large scale [93]. To improve the expression level of ANP and reduce the cost, three copies of ANP genes were fused in tandem to express the fusion protein His₆-K-ANP-K-ANP-K-ANP (His₆-ANP₃) in *E.coli* [93] [Fig. 4]. ANP can be obtained after digestion by Endoproteinase Lys-C and Carboxypeptidase B (CPB) and then purified by a series of purification processes. The purity of the protein was over 90% as determined by Tricine-SDS-PAGE and HPLC. LC-MS results also showed that the target protein was monomer ANP and had no obvious oxidation or deamination modification [93].

Construction of natriuretic peptide chimera

Chen et al. [94] designed a new 28 amino acid chimeric peptide called AC-NP, which combined the 17-amino acid ring of C type natriuretic peptide (CNP) with the 6-amino acid N-

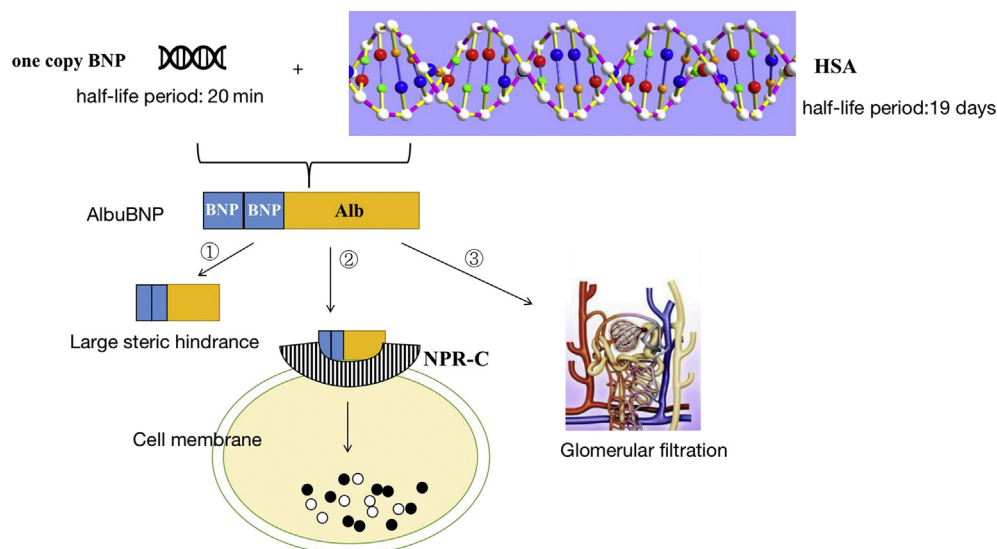


Fig. 5 The fusion of BNP and human serum albumin reduces proteolysis through three clearance pathways. ① The Large steric hindrance of BNP, so the elimination half-life of AlbuBNP in mice is 19 h, which is a dramatic increase from 3 min for BNP; ② Mediated with NPR-C receptor; ③ Glomerular filtration BNP.

terminus and 5-amino acid C-terminus of atrial natriuretic peptide (ANP) [Fig. S2A]. The *in vitro* and *in vivo* experiments were performed to determine the functions of AC-NP. In normal rats, AC-NP proved to be more potentially diuretic, natriuretic and hypotensive compared with other NPs [94]. In summary, the innovatively designed AC-NP may be a new candidate therapeutic peptide for natriuretic peptides in the future. The Cardiorenal laboratory first reported CD-NP in 2008 [95], CD-NP is a chimera of mature human CNP that consists of 37 amino acid. The 15 amino acid C-terminal of DNP is combined with CNP [Fig. S2B]. CD-NP acts on the GC-A and GC-B receptors of target cells [95]. The goal of the research is to design, synthesize and test *in vivo* and *in vitro* a new chimeric peptide that would combine the beneficial properties of 2 distinct natriuretic peptides with a biological profile that goes beyond native peptides.

Vasonatrin peptide is an artificial synthetic member of the NP family that is a chimera of CNP and ANP, is characterized by the 22-amino-acid ringed structure of CNP along with the COOH terminus of ANP [Fig. S2C]. As a synthetic natriuretic peptide, Vasonatrin peptide has cardiovascular bioactivity similar to the natural natriuretic peptides, ANP and CNP [96].

Construction of fusion proteins

Genetic engineering of natriuretic peptides is a relatively cheap method, but it is still in its infancy. The main difficulty is that the molecular weight of polypeptides is smaller than that of proteins. They are unstable after the expression in cells and are easily degraded. In order to increase their stability, the polypeptide can be fused with the carrier protein which then could be expressed in the prokaryotic system or eukaryotic

system as a fusion protein. Finally, the fusion protein is cut to obtain the target polypeptide [97].

Albumin is a monomeric macromolecule and the most abundant plasma protein. It can maintain plasma osmotic pressure and act as a regulator of fluid distribution in the average human body [97]. Simultaneously, human serum albumin (HSA) has a variety of physiological and biochemical functions. The inherent biochemical and biophysical properties of HSA, such as high abundance, stability, long-circulating half-life, and binding ability, make it an ideal drug delivery platform [Fig. 5]. A recombinant fusion of BNP with human serum albumin can stabilize BNP *in vivo* [98]. Simultaneously, to increase the biological activity of the fusion protein, the BNP(1–32) part was copied to create BNP(2x)/HSA. The fusion of BNP and HSA can reduce the cleavage of proteolytic BNP through steric hindrance, NPR-C receptor-mediated clearance, and renal small protein filtration of BNP (36–43) [Fig. 5]. Studies have shown that AlbuBNP is biologically active, has significantly improved pharmacokinetics in cardiovascular aspects, and is used for subsequent anti-tumor research through recombinant fusion into HSA [97].

Natriuretic peptides in combination with other drugs

Rhabdomyosarcoma (RMS) is a malignant stromal tumor and the most common soft tissue sarcoma in children, which requires novel therapies with less toxicity. CNP is an endogenous peptide secreted by endothelial cells, exerts antiproliferative effects in multiple types of mesenchymal cells. Masahiro Zenitani et al. [99] found CNP and sildenafil synergistically inhibited proliferation of RMS cells [Fig. 6] by inhibiting the Raf/MEK/ERK pathway, and decreased Raf-1, Mitogen-activated protein

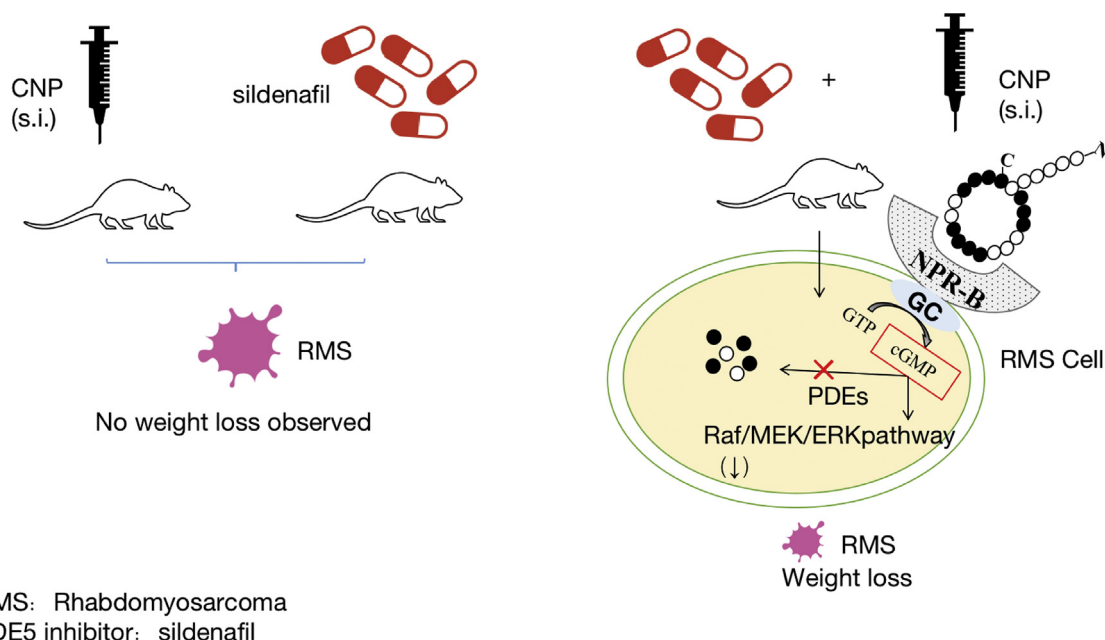


Fig. 6 CNP and sildenafil synergistically inhibit the growth of RD-GC-B xenograft tumors. Sildenafil as a selective PDE5 inhibitor, is added to inhibit its degradation *in vitro*. The combination of CNP and sildenafil can inhibit the Raf/MEK/ERK pathway and exert an anti-proliferative effect on RMS cells.

kinase kinase (MEK), and extracellular signal-regulated kinase (ERK) phosphorylation *in vitro* [99][Table 1].

Conclusions

In this context, we reinforce the validity of NPs as antitumor drug. The anti-tumor activity of NPs is mainly related to its interaction with specific receptors, NPRs, through inhibiting some signaling pathways crucial for cancer development, such as the RAS-MEK1/2-ERK1/2, Wnt/ β -catenin, or VEGF/VEG-FR2 signal pathways. We also introduced Tandem expression technique, designation of new peptides by fusion with a backbone protein, which could enhance the stability of NPs. In the future, natriuretic peptides might yield novel therapeutic modalities with greatly improved efficacy and patient compliance.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgment

This work was supported by National Natural Science Foundation of China (Grant No. 81802975), Zhejiang Provincial

Natural Science Foundation (Grant No. LY18H180010), Basic Public Welfare plan of Zhejiang Province (Grant No. LY21H160001) and Medical Health Science and Technology Project of Zhejiang province (Grant No. 2019KY361, 2020KY101).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bj.2021.06.007>.

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