

10-4-4, A Cardiac Glycoside Block Growth in Cancer Cells via Nuclear Receptor Nur77 Mediated Na⁺/K⁺-ATPase Endocytosis

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Abstract

Background

Cancer is second only to heart disease as a cause of death. Develop new and more effective treatment strategies for cancer remain a major challenge for human medicine today. Nur77, an orphan member for the nuclear receptor superfamily, inhibits growth in cancer cells by translocation to cytoplasmic. Small molecules that trigger Nur77 nuclear export may be an ideal anti-cancer candidate.

Methods

Cell proliferation was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The protein expression level were detected by western blot analysis. Immunostaining and cell fractionation assays were used to assess subcellular localization of Nur77. Cell apoptosis, cell cycle and calcium were detected by flow cytometry. Zebrafish liver cancer models were used to determine anti-cancer effect of 10-4-4 *in vivo*.

Results

In this study, we exhibit 10-4-4 a cardiac glycoside, extracted from *Antiaris toxicaria lesch*, has sensitivity to cancer cells. 10-4-4 induces apoptosis and G₂/M cell cycle arrest in HepG2 cells. Consistently, 10-4-4 augments Nur77 expression and cytoplasmic localization, its restraint of cancer cells growth is Nur77 dependent. Meanwhile, as a cardiac glycoside, 10-4-4 inhibits Na⁺/K⁺-ATPase (NaK) activation. To further confirm the molecular mechanism of 10-4-4, we found the association between Nur77 and NaK. The suppression of NaK by 10-4-4 increases the level of intracellular Ca²⁺. Ca²⁺, as a second messenger, specific activates protein kinase C (PKC). PKC has been reported on the influence of Nur77 nuclear export. Identical conclusions are obtained in this studies that 10-4-4 induces PKC activation play an important role in Nur77 nuclear export. Notably, the cytoplasmic Nur77 induced by 10-4-4 interaction with NaK to induce NaK endocytosis, and then trigger G₂/M cell cycle arrest and apoptosis. Studies in Zebrafish shows that 10-4-4 potently inhibits the growth of liver cancer cells *in vivo*.

Conclusions

Our results exhibit that 10-4-4 possesses an anti-cancer activity *in vitro* and *in vivo* via NaK-Nur77 signaling pathway and maybe offers a novel strategy in development of chemotherapeutic anti-cancer drug.

Backgroud

Cancer is the second leading cause of death globally[1]. Although various methods have been tried to study and treat cancer, the cancer remains a major challenge for human medicine today[1–3]. The poor 5-year relative survival rate, poor prognosis and resistance of tumors to chemotherapy warrants further investigation of the molecular mechanism of tumors and exists an unmet need for innovative approaches [4–7].

Nur77 (also known as TR3, NGFI-B, NR4A1, TIS1 and NAK-1) is a member of the nuclear receptor family and belongs to the Nur77 orphan receptor superfamily [8, 9]. Nur77 is regulated by a variety of intracellular or extracellular stimuli, plays important roles in numerous diverse biological processes including cell proliferation, differentiation, inflammation, survival and apoptosis [10–16]. Nur77 is highly expressed in different types of cancers. As an early immediate-response gene, Nur77, is rapidly expressed in cancer cells under the induction by a variety of growth factors and mitogens [17, 18]. Therefore, augmenting the expression level of Nur77 serves to maintain the growth and survival of cancer cells. Knocking down Nur77 from cancer cells attenuates their growth and enhances their apoptosis [18, 19]. Paradoxically, the expression of Nur77 is required for apoptotic effect of numerous apoptotic stimuli, including chemotherapeutic agents [18, 20]. Mechanistically, the mitogenic and survival effect of Nur77 appears to be dependent on its transcription regulation in the nucleus, while its pro-apoptotic effect involves its translocation from the nucleus to the cytoplasm [15, 16, 21–25]. Anti-cancer effect of many compounds is related to the apoptotic pathway after nuclear translocation of Nur77 [11, 12, 14–16, 23, 26–32]. As Nur77 is often higher expressed in cancer cells, contributing to their development and resistance to radiotherapy and chemotherapy [33–36], targeting Nur77 may serve as an attractive approach to overcome the cancers.

Natural products provide a rich source for the development of new bioactive small molecules and therapeutic agents for the treatment of a number of diseases including cancer [37, 38]. Our laboratory has been committed to the identification of natural compounds targeting Nur77 from Chinese herbs [39–42]. *Antiaris toxicaria* Lesch, a tropical plant widely found in Southeast Asia, contains a complex mixture of cardiac glycosides (CGs), which are used to treat congestive heart failure and arrhythmia [43]. It is commonly known that CGs target to α subunit of the Na^+/K^+ -ATPase (NaK) to control the flow of Na^+ and K^+ and Ca^{2+} ions in excitable cells [44, 45]. Over the years, an enormous amount of evidences have accumulated *in vitro* and *in vivo*, suggesting that CGs are also effective in the prevention and/or treatment of proliferative diseases, such as cancer [46, 47]. CGs have inhibitory effects on lung cancer [48–51], melanoma [22, 52], hepatocellular carcinoma [53, 54], glioblastoma [55], breast cancer [36, 37] [38], prostate cancer cells proliferation [56–58] and colon cancer [59, 60]. However, the mechanism of inhibition have not yet been clearly realized. Our previous studies have found that CGs induce cytoplasmic Nur77 to inhibit cancer cell growth [61] [42]. In this study, we report our finding that Cardiac glycosides 10-4-4, a GCs isolated from *antiaris toxicaria lesch.*, could potentially inhibit the growth of cancer cells. The death effect of 10-4-4 must rely on Nur77. As a cardiac glycoside, 10-4-4 activity is also closely related to NaK. Key to this paper is the finding that the association between Nur77 and NaK in 10-4-4 induced cell death. As we all know, cardiac glycoside 10-4-4 inhibits NaK activity to augment intracellular Ca^{2+}

accumulation. 10-4-4 up-regulated Ca^{2+} enhance PKC activity to induce nuclear export of Nur77. Cytoplasmic Nur77 interact with NaK $\alpha 1$ subunit to induce NaK endocytosis, increase apoptosis and G_2/M cell cycle arrest of cancer cells. In summary, NaK-Nur77 pathway, we observed here, is supposed to be the key role in Cardiac glycoside 10-4-4 induced cell death. 10-4-4 have great potential as an anti-cancer candidate with a new mechanism.

Materials And Methods

Reagents

10-4-4 was dissolved in dimethyl sulfoxide (DMSO, Sigma), and stored as a stock solution at 1 mg/mL at -20°C . It was diluted in cell culture medium or other buffer at indicated concentration when treated. The reagents used in the experiment were described as follows: p38 MAPK inhibitor (SB203580), PKC inhibitor (Go 6983), endocytosis inhibitor (Pitstop 2) were purchased from MedChemExpress.

Cell culture and Transient Transfection

In this study, human HCC cell lines HepG2, Huh-7, Hep3B and human liver cell line LO2 were purchased from Cell Resource Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China). HepG2, Huh-7 and LO2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM); NCI-H460 were cultured in 1640 medium; Hep3B were cultured in MEM medium, supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotics (100 U/ml of penicillin and streptomycin, Omega Scientific) at 37°C in a humidified atmosphere containing 5% CO_2 . Transient transfection was performed using Lipofectamine 2000 (Invitrogen) or RNAiMax transfection reagent (Invitrogen) according to the manufacturer's instructions.

Plasmids

pCMV-myc-Nur77, pGFP-Nur77AB, pGFP-Nur77 Δ AF2, pGFP-Nur77 Δ AB, pGFP-Nur77 Δ DBD, pGFP-Nur77 Δ AB/ Δ AF2, pGFP-Nur77LBD, pGFP-Nur77DC3, pGFP-Nur77N150, pGFP-Nur77 were described previously or were constructed using PCR[16].

siRNA and transfections

The human Nur77-SiRNA, human NaK $\alpha 3$ subunit-SiRNA and control siRNA used were from Sigma. Transfections were performed with RNAiMax transfection reagent (Invitrogen), according to the manufacturer's recommendations. Two days after transfection, the cells were treated with 10-4-4 for 3 hr (for protein expression analysis) or 12 hr (for apoptosis analysis), then harvested.

Cell proliferation assay

Cells were seeded at 3,000 cells per well in 96-well plate. After 24 hr of incubation, they were treated with 10-4-4 containing 10% FBS for 3 days. The control cells received vehicle containing equivalent DMSO.

Viable cells were determined by the addition of MTT ([3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium]–phenomenon methosulfate, Sigma) solution to each well, and a sequential incubation for 4 hr at 37°C in the dark. Absorbance value was measured at 550nm by a Bio-Rad 550 micro-plate reader. The results were represented as IC₅₀ (50% inhibiting concentration).

Flow Cytometry assay

Cells were collected by centrifugation at 1,000 rpm for 5 min, then resuspended cells with PBS and incubated with PI, Annixn V, and Flou-3AM for 1 hr and analyzed by flow cytometry (FCM), using the FACScater-plus flow cytometer (Becton Dickinson) to test its cell cycle, apoptosis and calcium.

Western blotting

After extraction, proteins in cell lysates were separated using SDS-PAGE. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. 5% non-fat milk was used to blocking nonspecific sites of membranes, then membranes were incubated with PARP, phospho-chk1, phospho-chk2, phospho-cdc2, Cyclin B1, Nur77, phospho-PKC, phospho-Akt, phospho-JNK, phospho-p38, Myc, NaK, GFP and β -actin antibodies: Rabbit monoclonal anti-PARP (Cell Signaling Technology, Cat# 9542, 1:1000 dilution), Rabbit monoclonal anti-phospho-chk1 (Cell Signaling Technology, Cat# 12302, 1:1000 dilution), Rabbit monoclonal anti-phospho-chk2 (Cell Signaling Technology, Cat# 2197, 1:1000 dilution), Rabbit monoclonal anti-phospho-cdc2 (Cell Signaling Technology, Cat# 4539, 1:1000 dilution), Rabbit monoclonal anti-Cyclin B1 (Cell Signaling Technology, Cat# 12231, 1:1000 dilution), Rabbit monoclonal Nur77 (Cell Signaling Technology, Cat# 3960, 1:1000 dilution), Rabbit monoclonal phospho-PKC (Cell Signaling Technology, Cat# 38938, 1:1000 dilution), Rabbit monoclonal phospho-Akt (Cell Signaling Technology, Cat# 13038, 1:1000 dilution), Rabbit monoclonal phospho-JNK (Cell Signaling Technology, Cat# 9251, 1:1000 dilution), Rabbit monoclonal phospho-p38 (Cell Signaling Technology, Cat# 9215, 1:1000 dilution), Rabbit monoclonal phospho-p38 (Cell Signaling Technology, Cat# 9215, 1:1000 dilution) antibodies were purchased from Cell Signaling Technology (Massachusetts, USA). Mouse monoclonal anti-Myc (Santa Cruz Biotechnology, Cat# sc-40, 1:2000 dilution), Mouse monoclonal anti-GFP (Santa Cruz Biotechnology, Cat# sc-9996, 1:2000 dilution) antibodies were purchased from Santa Cruz Biochemical (Santa Cruz, CA). Mouse monoclonal anti- β -actin (Sigma, Cat# A5441, 1:10000 dilution) antibody was purchased from Sigma (St. Louis, MO). Na⁺/K⁺-ATPase α 3 (ab2826, 1:1000) antibody was purchased from abcam (Abcam Inc. Cambridge, UK). Subsequently, the membranes were incubated with a HRP conjugated secondary antibody (Protein Tech Group, Chicago, IL) at room temperature for 1 hr. HRP-conjugated Goat Anti-Mouse IgG (H+L) (Protein Tech Group, Cat# SA00001-1, 1:10,000 dilution), HRP-conjugated Goat Anti-Rabbit IgG(H + L) (Protein Tech Group, Cat# SA00001-2, 1:10,000 dilution). The signals were observed by an enhanced chemiluminescence system (GE Healthcare; Munich, Germany).

Cell fractionation[23]

Cells were re-suspended in buffer A [10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM Dithiothreitol (DTT)], centrifuge the cell suspension at 6,000×g for 30 sec to collect cytoplasmic fraction. Re-suspend pellets in Buffer B [20 mM HEPES-KOH (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 0.5 mM DTT]. Centrifuge the supernatant at 12,000×g at 4°C for 15 min.

Co-immunoprecipitation (Co-IP) assays [15, 16, 23, 62]

The protein concentration in cell lysates was diluted to about 1 µg/µL, appropriate antibody was added into 500 µL total protein lysate, incubate for 2 hr. 20 µL protein A-Sepharose beads were added to capture the antigen-antibody complex, then detection of interacting proteins by western blotting.

Immunofluorescence microscopy [23]

Cells mounted on coverslips were fixed with 4% paraformaldehyde for 10 min; incubated the coverslips for 15 min with 0.1% Triton X-100 to permeabilize the cells. Incubated cell with 1% bovine serum albumin for 30 min at room temperature. Incubated cells with diluted primary antibodies in a humidified chamber for 1 hr and detected with fluorophore conjugated secondary antibodies. The images were taken under an LSM-510 confocal laser scanning microscope system (Carl Zeiss, Oberkochen, Germany).

Na⁺/K⁺-ATPase -ATPase activity assay

After treating cells with compounds, assayed NaK -ATPase activity of cell lysis with NaK activity Kit from Beyotime Institute of Biotechnology in China.

Zebrafish husbandry, Chemical treatments [63] and Photography and image analysis [63]

Transgenic line, Tg (fabp10:rtTA2s-M2; TRE2: EGFP-krasV12) (gz32Tg) in a Tet-On system to control the hepatocyte-specific expression of oncogenic kras^{V12} [64], was used in this study.

Doxycycline (Dox) (Sigma, D9891) was added at dose of 75 µg/mL from 3 to 7dpf to induce kras expression. The larvae were exposed to 5 µM 10-4-4 at 4 to 7 dpf. 20 larvae were randomly selected from each group for imaging. The larvae were anesthetized in 0.08% tricaine (Sigma, E10521) and immobilized in 3% methylcellulose (Sigma, M0521). Each larva was photographed with Olympus microscope (DP72). ImageJ was used to 2D measure the liver size.

Statistical analysis

Data were expressed as mean±SD. Each assay was repeated in triplicate in three independent experiments. Statistical significance of differences between groups was analyzed by using Student's t test or ANOVA analysis. p<0.05 was considered significant.

Results

The growth inhibitory effect of Cardiac glycoside 10-4-4 in cancer cells.

Studies have shown that some CGs may have anti-cancer effects. To identify new anti-cancer CGs, we screened more than 100 CGs, and found that 10-4-4 (Fig. 1A) could potentially inhibit the growth of cancer cells (data not show). We employed MTT assay to explore the effect of 10-4-4 on the growth of several cancer cell lines in this study (Fig. 1B and Table 1). 10-4-4 showed a great inhibition in Hepatocellular Carcinoma (HCC) cell lines, including HepG2, Huh-7, and Hep3B cells, with IC₅₀ values ranging from 20 to 27 nM, while it effects of the growth of H460 lung cancer cells with an IC₅₀ of 32.17 nM. 10-4-4 was ineffective in LO2, a normal liver cell line, with an IC₅₀ value greater than 791.6 nM (Fig. 1B and Table 1). Hence, 10-4-4 is effective against cancer cells but not normal cells.

Table 1
IC₅₀ of 10-4-4 Growth Inhibition in cancer cell lines

Cell line	Cell type	IC ₅₀ (nM)
HepG2	hepatocellular carcinoma	20.14
Hep3B	hepatocellular carcinoma	22.61
Huh-7	hepatocellular carcinoma	26.85
H460	Large cell lung cancer	32.17
LO2	Liver cell	791.6

Apoptosis and G₂/M phase arrest induction by Cardiac glycoside 10-4-4.

To further characterize the effect of 10-4-4 on cancer cell growth inhibition, we used flow cytometry (FCM) to examine its effect on apoptosis and cell cycle arrest in HepG2 cells, which was the one of the most sensitive cell lines among cancer cell lines were analyzed (Table 1). Annexin V/PI staining showed that about 72.6% of HepG2 cells had extensive early apoptosis (Annexin V⁺/PI⁻) after 20 nM 10-4-4 treatment for 24 hr. The apoptotic effect of 40 nM 10-4-4 on HepG2 cells was enhanced, especially the induction of Annexin V⁺/PI⁺ cells from 19.9–69.0% (Fig. 2A). Furthermore, our analysis of nuclear morphology by DAPI staining demonstrated that HepG2 cells treated with 10-4-4 for 12 hr caused morphological changes characteristics of apoptosis, including nuclear condensation and fragmentation, Ouabain used as a positive control (Fig. 2B). The apoptotic effect of 10-4-4 was also substantiated by its induction of the cleaved poly ADP-ribose polymerase (PARP) in a dose dependent manner (Fig. 2C).

Furthermore, we found that 10-4-4 could significantly augment G₂/M phase arrest in HepG2 cells (Fig. 2D). This was validated experimentally by utilizing western blotting to detect the accumulation of cell cycle regulators (Fig. 2E). We did observe the accumulation of phosphorylated Chk1, Chk2 and Cdc2 at 12 hr and 24 hr 10-4-4 treatment (Fig. 2E). At the same time points, we also observed a significant

increase in the level of cyclin B1. These results indicate treated with 10-4-4 result in the G₂/M phase arrest in HepG2 liver cancer cells.

Role of Nur77 expression and cytoplasmic translocation for its apoptosis and G₂/M phase arrest induction.

Our previous studies purported that some Cardiac glycosides acted as Nur77 modulators [39, 42, 61]. In order to trace whether correlation between growth inhibitory effect of 10-4-4 and Nur77 expression, we first studied its effect on Nur77 expression in HepG2 cells. Time course effect of Nur77 induction by 10-4-4. Western blot (Fig. 3A) analyses showed that 10-4-4 induction of Nur77 expression was clearly seen after cells were treated with the compound for 2 hr, the maximum induction occurred at 3 hr. The same phenotype was observed in Huh-7 cells (Fig. 3B). The time courses of Nur77 induction by 10-4-4 in HepG2 cells and Huh-7 cells were different with that observed with retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437) [38, 45] and acetylshikonin and analogues [14]. Suggest 10-4-4 may restraint of cancer cells growth by a new mechanism. Meanwhile, 10-4-4 also induced Nur77 expression in a dose-dependent manner, significant Nur77 induction was observed when 20 nM or higher dose of 10-4-4 was used (Fig. 3C).

Because translocation of Nur77 from the nucleus to cytoplasm plays a critical role in mediate its apoptotic effect [22, 23, 65], we examined whether Nur77 migrated to cytoplasm after 10-4-4 treatment. Under normal culture conditions, the expression of Nur77 protein in cells was low. HepG2 cells treated with 10-4-4 showed strong Nur77 staining, and Nur77 was distributed in both nucleus and cytoplasm (Fig. 3D). In order to confirm the inducing effect of 10-4-4 on Nur77 and its cytoplasmic localization, the cells were treated with 10-4-4 and the cells were separated into two distinct fractions : cytoplasmic and nuclear. The level of Nur77 protein in each fractions could then be analyzed by western blotting. In the control cells, Nur77 was mostly expressed in the nucleus. After the cells were treated with 10-4-4, in cytoplasmic fraction of HepG2 cell, Nur77 protein level was significantly increased (Fig. 3E). These results demonstrated that 10-4-4-induced Nur77 is predominantly cytoplasmic.

To determine the role of Nur77 expression to the apoptotic effect of 10-4-4, HepG2 cells were transfected with Nur77 siRNA and evaluated for its effect on apoptosis and G₂/M phase arrest induction by 10-4-4. Our results showed that the apoptotic effect of 10-4-4 measured by its induction of PARP cleavage was attenuated upon transfection of Nur77 siRNA (Fig. 3F). Transfection of Nur77 siRNA significantly diminished the G₂/M phase arrest effect of 10-4-4 (Fig. 3G). Expression levels of phosphorylated Chk1, Chk2, Cdc2 and Cyclin B1 induced by 10-4-4 were also inhibited (Fig. 3H). These results demonstrated that 10-4-4 induced apoptosis and G₂/M phase arrest is Nur77 dependent.

Cardiac glycoside 10-4-4 inhibit NaK activity to induce Nur77 nuclear export.

Since cardiac glycosides always worked as NaK inhibitors, NaK activity was measured in HepG2 cells treated with 10-4-4, Ouabain and Digoxin were positive controls. 10-4-4 had obvious decrement of NaK,

which was the normal reaction of common CGs like Ouabain and Digoxin (Fig. 4A). 10-4-4 showed a dose- and time-dependent inhibition of NaK (Fig. 4B). Meanwhile, HepG2 cells treated with Ouabain, Digoxin and 10-4-4 were stained with Fluo-4AM (used to measure Ca^{2+} concentrations inside living cells), and then measured intensity in 488nm. Ouabain increase 21.4%, Digoxin increase 5.5% and 10-4-4 could increase 7.7% calcium concentration in contrast to control (Fig. 4C). These results lead us to conclude that 10-4-4 is also a NaK inhibitor.

However, a major problem was if NaK inhibition effect of 10-4-4 was associated with Nur77 and cancer cell growth. We note that BAPTA-AM, a selective Ca^{2+} chelator could not inhibit 10-4-4 induced Nur77 expression (Fig. 4D) but could significant inhibit 10-4-4 induced Nur77 nuclear export (Fig. 4E). However, calcium ionophore Ionomycin significantly enhanced the Nur77 nuclear export induced by 10-4-4. Suggesting Ca^{2+} play an important role in the induction of nuclear export of Nur77. Ca^{2+} is a well-known second messenger inside most of the cell types and can activate many protein kinases (MAPKs). MAPKs play an important role in the regulation of Nur77 [23, 66]. To determine whether MAPKs signaling pathway was involved in the induction of Nur77 nuclear export by 10-4-4, we studied the effect of 10-4-4 on the phosphorylation of the PKC, Akt, JNK and p38 (Fig. 4F). PKC was rapidly and strongly activated by 10-4-4, but we did not observe augmented levels of activation of Akt, JNK and p38 in HepG2 cells (Fig. 4F). PKC inhibitor could inhibit 10-4-4 induced Nur77 nuclear export, as a negative control, p38 inhibitor-SB200325 could not (Fig. 4G). Together, these results suggest that 10-4-4 inhibit NaK to increase intracellular Ca^{2+} to active PKC induce Nur77 nuclear export.

Cardiac glycoside 10-4-4 promotes Nur77 interaction with NaK.

In order to trace the localization of Nur77 induced by 10-4-4, immunostaining assay was performed. The results showed that cytoplasmic Nur77 colocalized extensively with NaK in cells treated with 10-4-4 (Fig. 5A). Similar result was detected in HepG2 cells overexpression of GFP-Nur77, when cells treatment by 10-4-4, overexpressed Nur77 also colocalized with NaK (Fig. 5B). The co-translocation of Nur77 and NaK induced by 10-4-4, prompting us to study whether Nur77 interacts with NaK. Co-immunoprecipitation (Co-IP) assay showed that 10-4-4 induced specific coimmunoprecipitated of Myc-Nur77 and NaK (Fig. 5C); Ouabain and Digoxin have no effect on Nur77 and NaK interaction (Fig. 5C). Thus, Nur77, upon induction of 10-4-4, interact with NaK. To further describe the interaction between Nur77 and NaK, we determined the Nur77 domain that interact with NaK. We constructed some Nur77 mutants with GFP tag. Analysis of these mutants revealed that the C-terminal, like LBD and DC3 of Nur77 was responsible for binding NaK. N-terminal (like N150) could not interact with NaK. (Fig. 5D-E).

Nur77 interaction with NaK triggers NaK endocytosis to induce cancer cell G₂/M arrest.

More interestingly, as showed in Figure 5A, NaK have different localization in control cells and 10-4-4 treated cells. In control cells, NaK most localized at plasmalemmal. In contrast, treated cells by 10-4-4, NaK diffused distribution in both the plasmalemmal and cytoplasm. CGs, as Ouabain could induces endocytosis of plasmalemmal NaK to inhibit the activation of the enzyme. To determine whether 10-4-4-

induced NaK endocytosis, cells treated with 10-4-4 were stained with NaK and EEA1, (an early endosome marker). Confocal microscopy showed that in the cells treated with 10-4-4, the distribution of NaK and EEA1 had a wide overlap (Fig. 6A). To confirm the effect of 10-4-4 on inducing NaK endocytosis, the cells were treated with 10-4-4 and the cells were separated into two distinct fractions: cytoplasmic and membrane. NaK expression level was analyzed by immunoblotting. In control cells, NaK was low in the cytoplasmic fraction. 10-4-4 treatment cells, cytoplasmic NaK increased dramatically (Fig. 6B). Suggesting that 10-4-4 could induce NaK endocytosis. We next determined whether Nur77 mediated the NaK endocytosis effect of 10-4-4. siRNA inhibit Nur77 impaired the ability of 10-4-4 to induce NaK endocytosis (Fig. 6C). These results show that the NaK endocytosis effect of 10-4-4 also depends on Nur77. To determine whether 10-4-4 induced cell cycle arrest dependent on NaK endocytosis, Pitstop 2, an endocytosis inhibitor was used to block endocytosis of NaK induced by 10-4-4 (Fig. 6D). Pitstop 2 could significantly decrease G₂/M arrest induced by 10-4-4 (Fig. 6E), suggest 10-4-4 induced G₂/M arrest was dependent on NaK endocytosis.

The inhibitory effect of Cardiac glycoside 10-4-4 on the growth of liver cancer.

To study the anti-cancer effect of 10-4-4 in animals, we use an inducible *kras*^{V12} transgenic zebrafish liver cancer model to investigate anti-liver cancer activity of 10-4-4 *in vivo*. After inducing the expression of Kras with doxycycline (Dox) to induce liver cancer, the *kras*⁺ larvae were incubated in 5 μ M 10-4-4 from 4 dpf to 7 dpf. In Dox only larvae (control), the expression of *kras*^{V12} oncogene significantly enlarged the size of the liver, which was round and spherical. The normal liver contour was restored after co-incubated with 10-4-4 and Dox (Fig. 7A). Two-dimensional measurement of liver size based on the GFP expression confirmed that Dox exposure significantly increased liver size of *kras*⁺ larvae, while co-incubation with 10-4-4 reduced the liver enlargement induced by *kras* oncogene (Fig. 7B). These results suggested that 10-4-4 could abrogate the oncogene-induced liver enlargement.

Discussion

Anti-cancer drug development remains one of the major challenges for global medicine. Several studies have revealed that the anti-cancer effect of cardiac glycosides. A number of inventions emphasize the potential usefulness of CGs can specifically inhibit the proliferation of cancer cells and induce apoptosis, but have no significant effect on normal cells [45]. Some CGs have entered clinical trials of cancer treatment [67]. However, uncertainty remains as to the mechanism by which cardiac glycosides results in cancer cells growth inhibition. In this study, we show that 10-4-4, a cardiac glycoside isolated from *Antiaris toxicaria* Lesch significantly inhibites the growth of cancer cells (Fig. 1B and Table 1). 10-4-4 induces cancer cells apoptosis and G₂/M arrest (Fig. 2) in a Nur77-dependent manner (Fig. 3F-H).

Nur77 (also called TR3 or NGFI-B) was a potent proapoptotic member of the nuclear receptor superfamily [8, 68]. Responded to different death agents, it often exported from nuclear to cytoplasmic to induce apoptosis of cancer cells. However, uncertainty remains as to the mechanism by which Nur77 mediated apoptotic pathway. Here, we observed that 10-4-4 robustly induces Nur77 protein expression in HCC cells

(Fig. 3A and B), and cytoplasmic localization (Fig. 3D and E) is associated with apoptotic and G₂/M cell cycle arrest effect of 10-4-4 (Fig. 3F-H).

It is generally accepted that cardiac glycosides were inhibitors of the NaK. NaK were membrane-bound enzymes. It uses ATP to transport Na⁺ and K⁺ to the cell membrane. Inhibition effect of CGs on NaK, lead to an elevation of intracellular sodium, which activated the Na⁺/Ca²⁺ exchanger, leading to an increase of intracellular calcium[69]. Identical conclusions are obtained in this study, as a CG, 10-4-4 also inhibits ATPase activity (Fig. 4A and B) and increases the intracellular Ca²⁺ (Fig. 4C).

One of the challenges in the anti-cancer effect of 10-4-4 is if it NaK inhibition activity is related to Nur77 nuclear export and cancer cell growth inhibition? Considering that, 10-4-4 attenuates NaK activity (Fig. 4A and B) leads to augment of intracellular Ca²⁺ (Fig. 4C). Ca²⁺ was a well-known second messenger in most cells, which could activate many protein kinases, include MAPK, PKC, and Akt, resulting in the proliferation, differentiation and promotion of autophagy or apoptosis[44, 59, 60, 70, 71]. Protein kinases played an important role in the regulation of Nur77 and cancer cell growth [11, 23, 26–28]. Ca²⁺ may have potential application in 10-4-4 induced Nur77 effect. To achieve a better understanding of this question, the levels of the Nur77, is examined by western blotting assay. What surprising is that intracellular Ca²⁺, does not affect Nur77 expression. As can be seen from part D of Figure 4, BAPTA-AM (a selective Ca²⁺ chelator) does not diminish 10-4-4 induced Nur77 expression level. It must be mentioned that increased Ca²⁺ by 10-4-4 significantly and specifically active PKC, but not other protein kinase such as Akt, JNK, and p38 (Fig. 4F). It has been reported that sufficient cytoplasmic localization of Nur77 requires Ca²⁺ and PKC signals [27]. In our study, BAPTA-AM, although, doesn't inhibit 10-4-4 induced Nur77 expression, but it significantly diminishes Nur77 nuclear export. In contrast, Ionomycin, an effective Ca²⁺ ionophore increases the cytoplasmic Nur77 (Fig. 4E). Multiple kinases, such as Akt, JNK, p38 MAPK has been reported to induce Nur77 nuclear export [11, 12, 23, 26, 29]. However, in this study, 10-4-4 dose not induce AKT, JNK, and p38 (Fig. 4F). p38 inhibitor SB203580 doesn't affect the subcellular localization of Nur77 proteins induced by 10-4-4 (Fig. 4G). Meanwhile, PKC inhibitor is sufficient to reduce of Nur77 nuclear export (Fig. 4G). These results are different from nuclear export translocation of Nur77 induced by other stimulators, such as CCE (p38)[23], CD437 (JNK and Akt)[11]. From the results we have obtained, one can conclude that 10-4-4 mediated a novel pathway to induce Nur77 nuclear export. 10-4-4 inhibits NaK to increase Ca²⁺ to active PKC, activation of PKC is required for cytoplasmic localization of Nur77.

Nur77 nuclear export play critical role of many apoptosis-inductors has been intensively investigated. Little was known, however, about cytoplasmic Nur77 mediated cancer cells growth inhibition pathway. Notably, in this study, PKC induces cytoplasmic Nur77 interact with NaK (Fig. 5). The mutant Nur77/LBD and Nur77/DC3, which mainly exists in the cytoplasm, interacte with NaK (Fig. 5D), and colocalize with NaK in the cytoplasm (Fig. 5E), demonstrates that Nur77- NaK interaction may not be involved in its transcriptional function [16]. Meanwhile, the results of immunostaining shows that different from control cells, in 10-4-4 treated cells, NaK is not only localized on the cell membrane, but also expresses in the cytoplasm (Fig. 5A). Many studies have demonstrated that the endocytosis of NaK in cells incubated

with non-toxic doses of ouabain and other CGs [72, 73], but the mechanism and function still unclear. In this study, we figure out that 10-4-4 induces NaK colocalization with EEA1 (Fig. 6A). Cytosolic and Membrane fractions of cells also shows that 10-4-4 reduce NaK on the cell membrane and increase NaK in the cytoplasm (Fig. 6B). Pitstop 2 an endocytosis inhibitor prevents co-localization of NaK and EEA1, demonstrates 10-4-4 induces endocytosis of NaK (Fig. 6D). Interestingly, 10-4-4 induces NaK endocytosis is dependent on Nur77. In Nur77 knockdown cells, 10-4-4 dose not induce NaK endocytosis (Fig. 6C). NaK endocytosis is an important event of 10-4-4 induced G₂/M arrest, for endocytosis inhibitor-Pitstop 2 inhibit the endocytosis of NaK also inhibits G₂/M arrest induced by 10-4-4 (Fig. 6E).

Conclusions

In this study, we investigated the mechanism of Cardiac glycoside 10-4-4 induced apoptosis and cycle arrest of cancer cells. Briefly, the growth inhibition effect of Cardiac glycoside 10-4-4 in cancer cells depended on its induction of Nur77 cytoplasmic localization. Interestingly, the activation of Nur77 pathway by 10-4-4 was associated with its inhibition of NaK. Restraint of NaK leads to an increase of intracellular calcium. Ca²⁺ act as second messenger to augment PKC activity, resulting in the nuclear export of Nur77. Cytoplasmic Nur77 interact with NaK α subunit to induce endocytosis of NaK, results in cancer cells apoptosis and G₂/M arrest (Fig. 8). Therefore, our findings provide new insight into the mechanism by which NaK and Nur77 mediate the growth inhibition effects of diverse chemotherapeutic agents for cancer cells. Our results also reveal of 10-4-4 may be a potential anti-cancer agent with high efficiency. This work has expanded the possibility of 10-4-4 represents a promising agent for cancer therapy that warrants further clinical development.

List Of Abbreviations

NaK, Na⁺/K⁺-ATPase; CGs, Cardiac glycosides; PKC, protein kinase C; FBS, fetal bovine serum; Dox, Doxycycline; dpf, days' post fertilization; DMSO, dimethyl sulfoxide; Co-IP, Co-immunoprecipitation

Declarations

Ethics approval and consent to participate

Informed consent was obtained from all participants. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Xiamen University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors have declared that no competing interest exists.

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Author contributions

Hu mengjie: Investigation, Writing- Original draft; Xu Yang: Investigation, Funding acquisition; Bao Yuzhou: Investigation; Tang Jinshan: Resources, Writing - Review & Editing; Li Mingyu: Resources; Yao Xin-Sheng: Supervision; Zhang Xiao-kun: Conceptualization and Supervision; Jie Liu: Conceptualization, Writing - Review & Editing.

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Figures

Figure 1

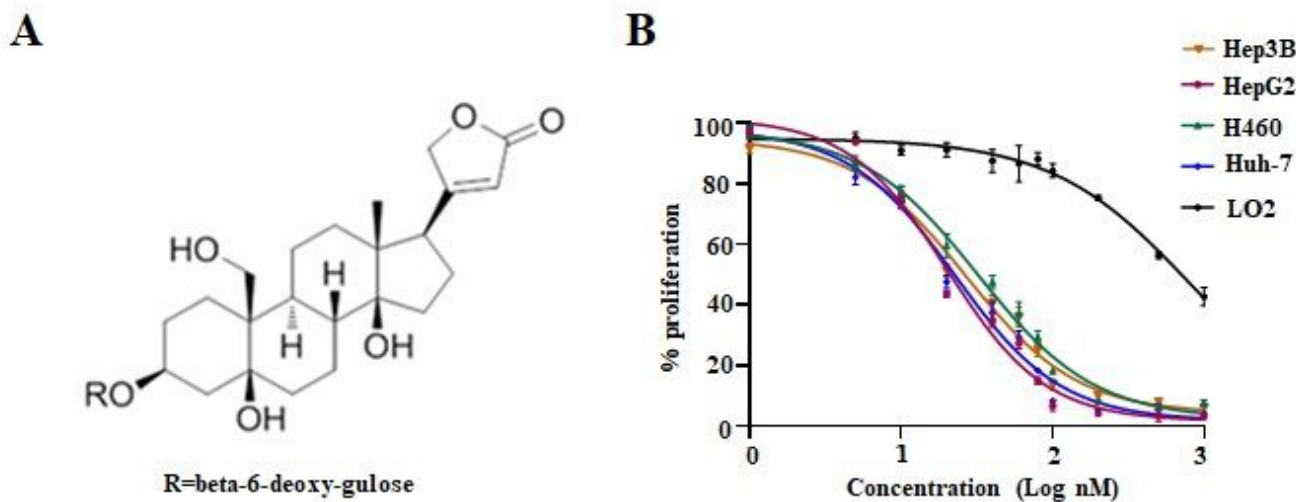


Figure 1

Growth inhibition of cancer cells by 10-4-4. A. Schematic structure of 10-4-4. B. Various cells, like HepG2, Hep3B, Huh-7, NCI-H460 and LO2 were treated with 10-4-4 with the indicated concentrations and cell proliferation was determined by MTT assay.

Figure 2

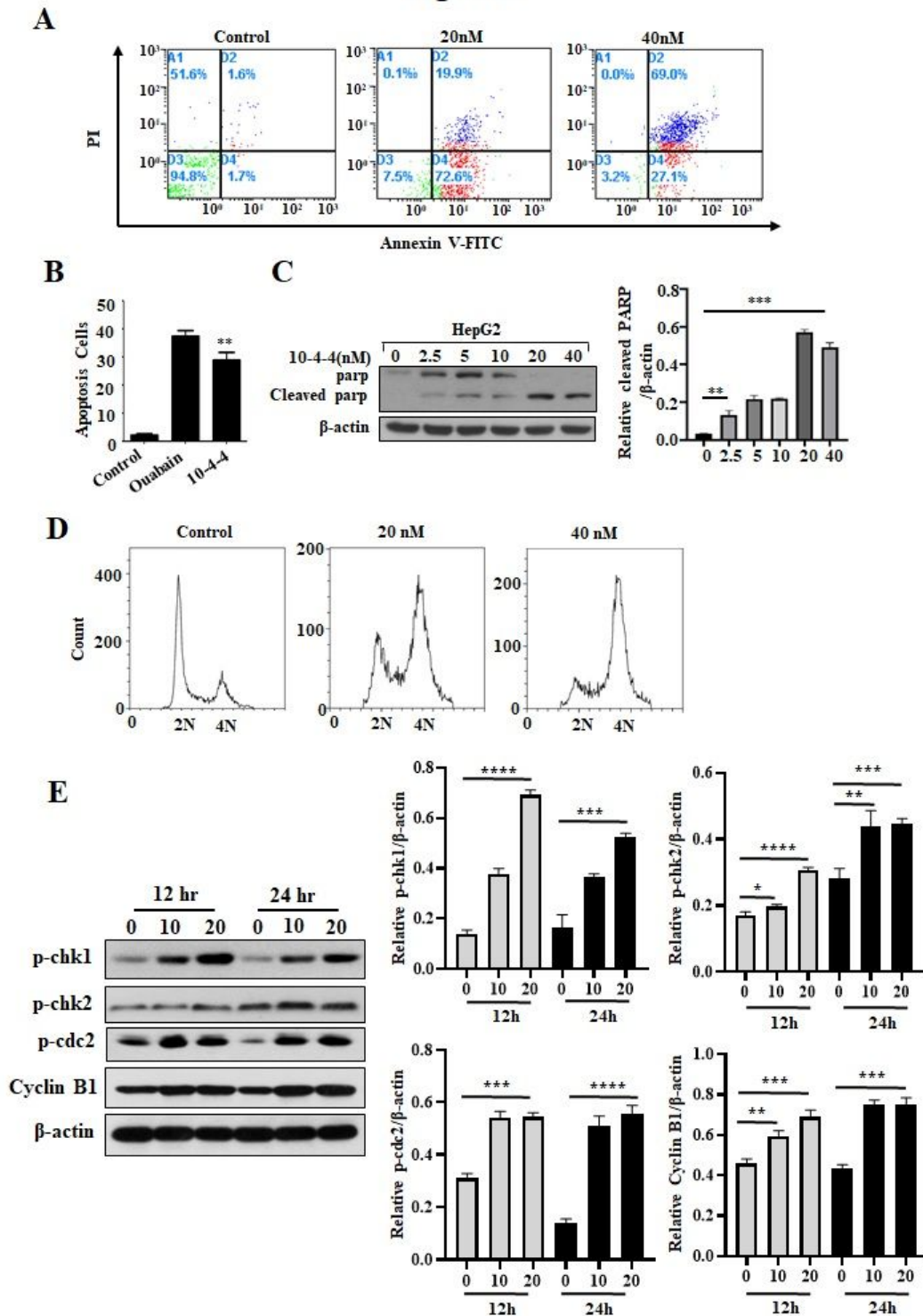


Figure 2

10-4-4 could induce apoptosis and G2/M phase arrest in cancer cells. A. Apoptosis induction was detected by FACS with PI and AnnexinV-FITC staining in HepG2 cells. HepG2 cells were treated at 20 nM and 40 nM of 10-4-4 for 24 hr. B. Cell morphology was analyzed by DAPI staining in HepG2 cells. Nuclear fragment was seen in the cells treated by 10-4-4 (20 nM) and Ouabain (20 nM) for 12 hr. C. Dose dependent apoptotic effect of 10-4-4. Western blotting analyzed PARP cleavage in HepG2 cells treated

with 10-4-4 for indicated concentration. Western blotting bands were quantified and shown in histogram. The data are expressed as the mean $\pm$ SEM; ** $P < 0.01$, *** $P < 0.001$. D. Cell cycle arrest was detected by FACS in HepG2 cells. Cells were treated with 10-4-4 (20 nM) for 12 hr stained with DAPI for detecting the cell cycle. E. G2/M transition-related proteins (phosphorylated (p-) chk1, phosphorylated (p-) chk2, phosphorylated (p-) cdc2, Cyclin B1) were analysis by western blot after 10-4-4 treatment of HepG2 cells for 12 hr and 24 hr. Western blotting bands were quantified and shown in histogram. Data are presented as mean $\pm$ standard deviation (SD) of three fields. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 3

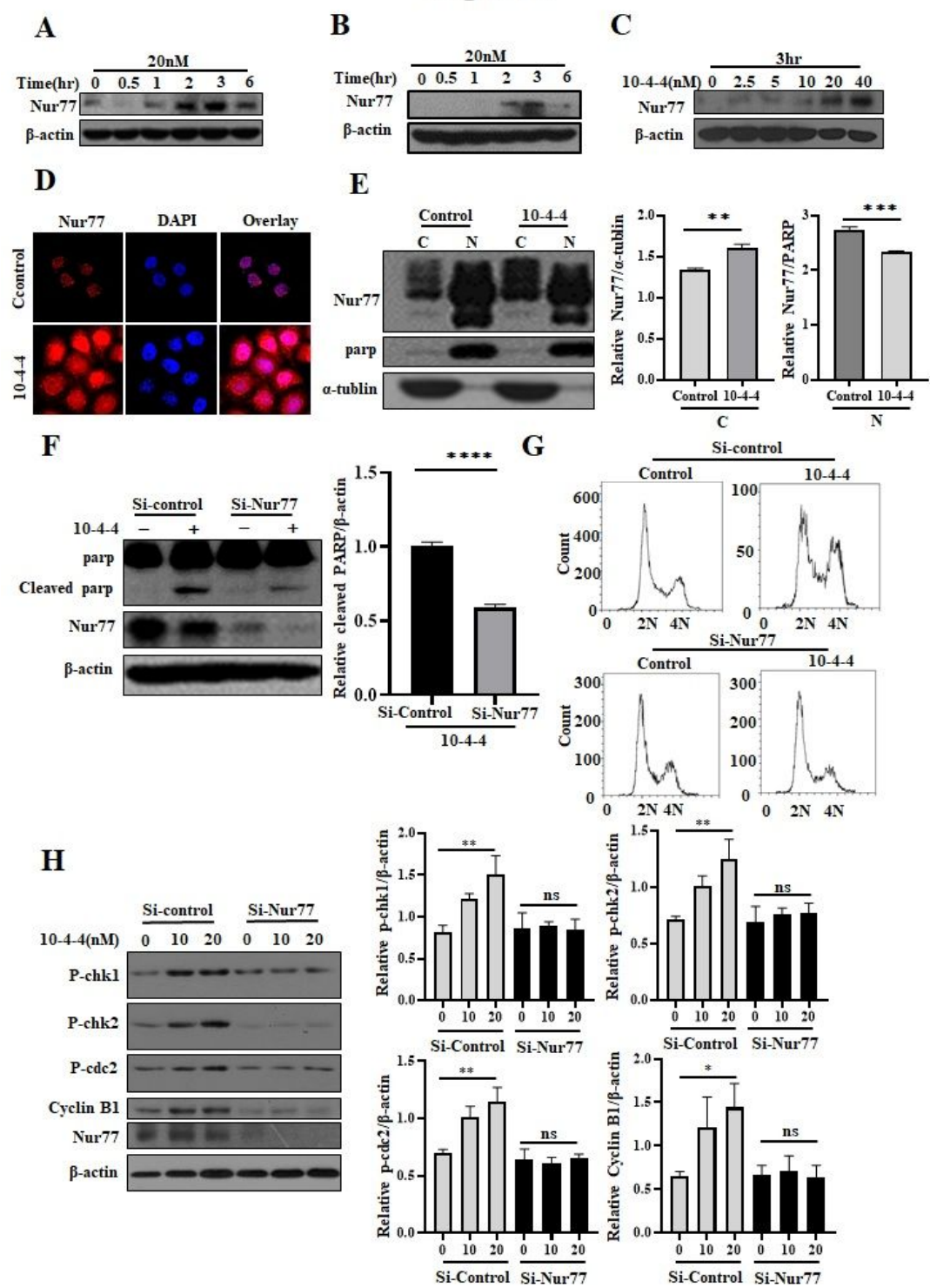


Figure 3

Nur77 was necessary for 10-4-4 induced apoptosis and G2/M phase arrest. A-B. Induction of endogenous Nur77 expression by 10-4-4 in time-dependent manner. Western blot analysis of the Nur77 expression in HepG2 cells and Huh-7 cells treated with 20 nM 10-4-4 for the indicated time. C. Dose-dependent analysis of Nur77 induction by 10-4-4. Western blot analysis of Nur77 expression of HepG2 cells treated with indicated dose 10-4-4 for 3hr. D. Nur77 nuclear export was measured by fluorescent immunostaining. HepG2 cells treated with 20 nM 10-4-4 for 3 hr were immunostained with Nur77 (red)-antibody and DAPI (blue). E. Cellular fractionation. Immunoblots for Nur77 in nuclear and cytoplasmic fractions of HepG2 cells by incubation of 20 nM 10-4-4 for 3 hr. PARP and Tubulin expression verified fraction purity. Western blotting bands were quantified and shown in histogram. F. 10-4-4 induced apoptosis was inhibited by transfection of Nur77 siRNA. WT and Nur77-silenced HepG2 cells were treated with 10-4-4 (20 nM) for 24 hr. The PARP cleavage were assayed using Western blotting. Western blotting bands were quantified and shown in histogram. G-H. Transfection of Nur77 siRNA inhibits cell cycle inhibition induced by 10-4-4. WT and Nur77-silenced HepG2 cells were treated with were treated with 10-4-4 (20 nM) for 12 hr and 24 hr. The protein exprssions of G2/M transition-related proteins were assayed using Immunoblot (G) and for analyzed cell cycle by flow cytometry (H), Western blotting bands were quantified and shown in histogram. Data are presented as mean $\pm$ standard deviation (SD) of three fields. **P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Figure 4

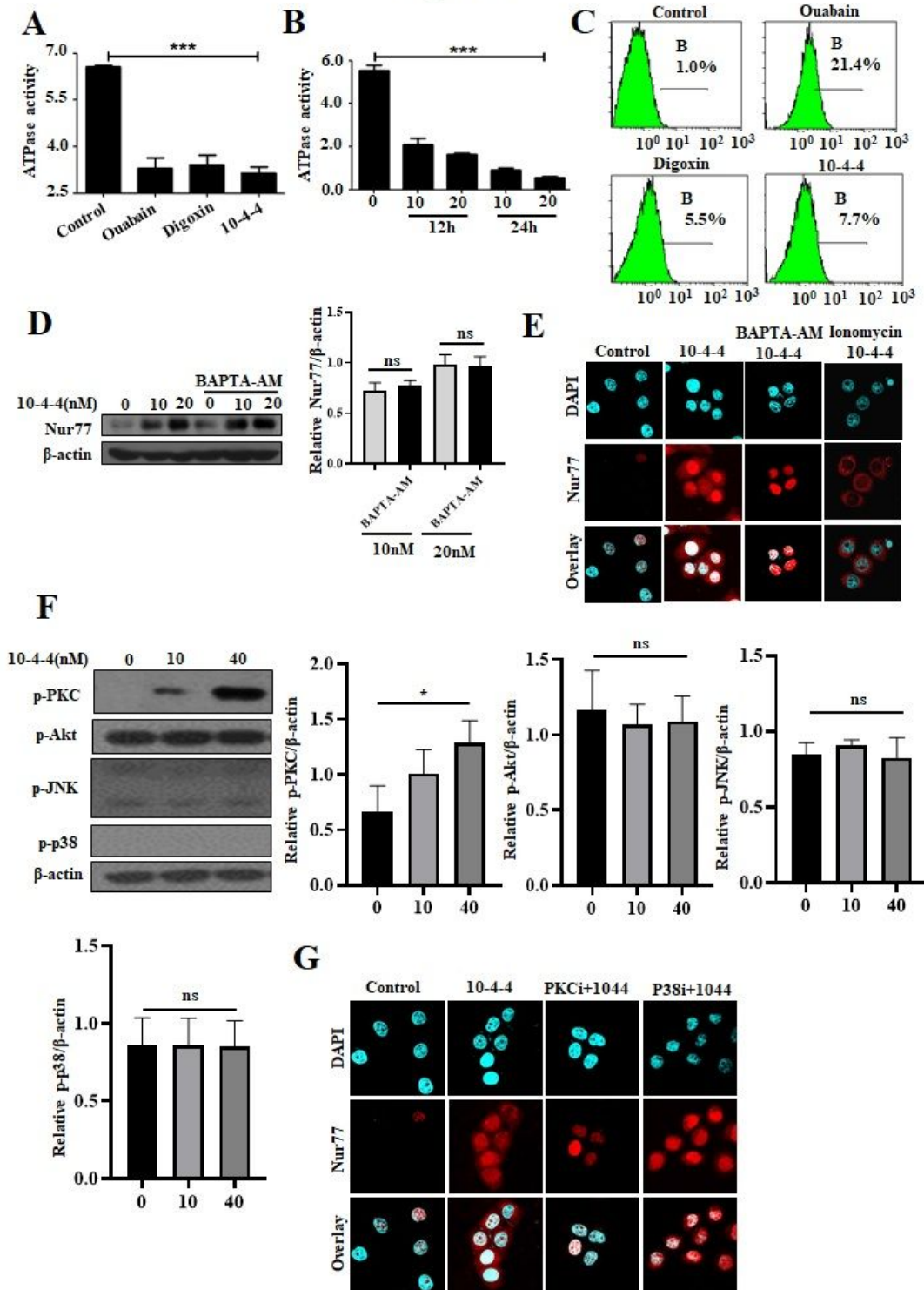


Figure 4

10-4-4 inhibit NaK activity to raise calcium ions lead to induce Nur77 nuclear export. A. Incubation of HepG2 cells with Ouabain (20 nM), Digoxin (20 nM), 10-4-4 (20 nM) for 12 hr and analyzed ATPase activity. B. HepG2 cells were treated with 10-4-4 (10 nM, 20 nM) for 12 hr and 24 hr and analyzed ATPase activity. C. HepG2 cells were treated with Ouabain (20 nM), Digoxin (20 nM), 10-4-4 (20 nM) for 12hr and analyzed Calcium by Fluo-3AM staining by flow cytometry. D. HepG2 cells were pre-treated by BAPTA-AM

for 1 hr and treated with 10-4-4 for 3 hr, Nur77 expression was analyzed by Western blotting, bands were quantified and shown in histogram. E. HepG2 cells were pre-incubated by BAPTA-AM and Ionomycin for 1 hr with 10-4-4 (20 nM) for 3 hr and analyzed for Nur77 translocation by confocal microscopy. F. 10-4-4 specifically induced PKC activation. HepG2 cells were treated with 10-4-4 (20 nM) for indicated time and analyzed phosphorylated (p-) PKC, phosphorylated (p-) Akt, phosphorylated (p-) JNK, phosphorylated (p-) p38 Western blotting, Western blotting bands were quantified and shown in histogram.. G. HepG2 cells were pre-treated by PKC inhibitor and p38 inhibitor for 1 hr with 10-4-4 (20 nM) for 3 hr and analyzed for Nur77 translocation by confocal microscopy. Data are presented as mean $\pm$ standard deviation (SD) of three fields. * $P < 0.05$, *** $P < 0.001$.

Figure 5

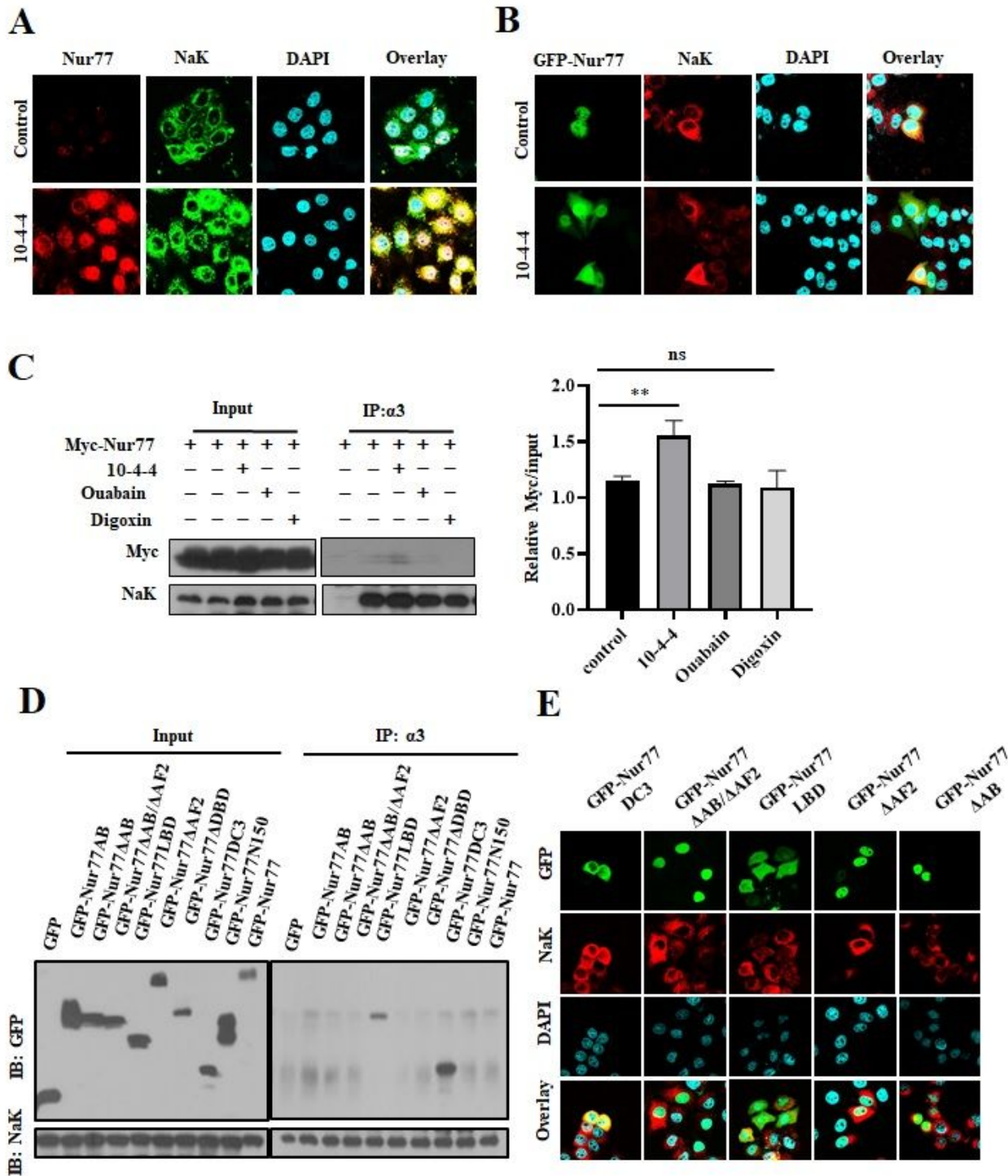


Figure 5

10-4-4 promotes Nur77 /NaK interaction. A. 10-4-4 induced co-localization of Nur77 and NaK. Incubation of HepG2 cells with 10-4-4 (20 nM) for 3h and analyzed Nur77 and NaK by confocal microscopy. B. HepG2 cells overexpressing GFP-Nur77, were treated with 10-4-4 (20 nM) for 6h and analyzed GFP-Nur77 and NaK by confocal microscopy. C. Vector- and Myc-Nur77-transfected HepG2 cells were treated with 10-4-4 (40 nM), Ouabain (40 nM), Digoxin (40 nM) for 3h and analyzed interaction of Myc-Nur77 and NaK by

immunoprecipitated (IP), Western blotting bands were quantified and shown in histogram.. D. GFP-WT- and different mutants-Nur77 transfected HepG2 cells were treated with 10-4-4 for 3h and analyzed interaction of Nur77 and NaK by immunoprecipitated (IP). E. GFP-WT- and different mutants-Nur77 transfected HepG2 cells were treated with 10-4-4 Nur77 for 3h and analyzed colocalization of Nur77 and NaK by immunostaining. Data are presented as mean $\pm$ standard deviation (SD) of three fields. ** P<0.01.

Figure 6

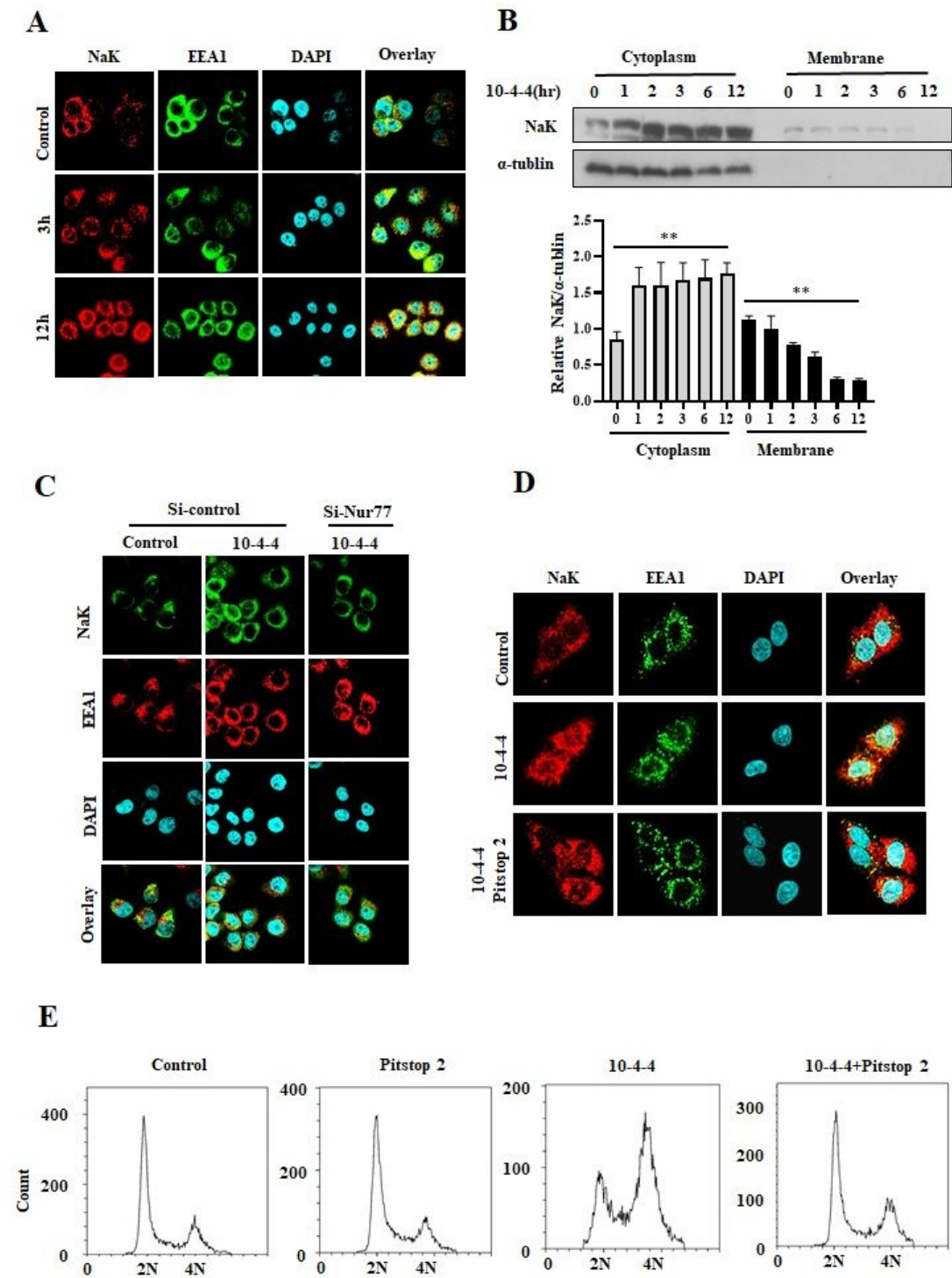


Figure 6

Nur77 triggers NaK endocytosis to induce cancer cell G2/M arrest. A. 10-4-4 induced co-localization of EEA1 and NaK. HepG2 cells treated with 10-4-4 (20 nM) for 3h and analyzed EEA1 and NaK by confocal microscopy. b. Cellular fractionation. Treatment of HepG2 cells with 20 nM 10-4-4 for indicated time to prepare cell membrane fractions and Western blot analysis were performed. Anti-Tubulin antibodies were used to identify the purity of cell components. Western blotting bands were quantified and shown in histogram. C. Transfection of Nur77 siRNA inhibits co-localization of EEA1 and NaK induced by 10-4-4. WT and Nur77-silenced HepG2 cells were treated with 10-4-4 (20 nM) for 3 hr and analyzed EEA1 and NAK by confocal microscopy. D. HepG2 cells pre-incubated with endocytosis inhibitor Pitstop 2 for 1hr and then treated with 10-4-4 (20 nM) for 3 hr, EEA1 and NaK localization were analyzed by confocal microscopy. E. HepG2 cells pre-incubated with endocytosis inhibitor Pitstop 2 for 1hr and then treated with 10-4-4 (20 nM) for 12 hr and analyzed cell cycle by DAPI staining by flow cytometry. Data are presented as mean $\pm$ standard deviation (SD) of three fields. ** $P < 0.01$.

Figure 7

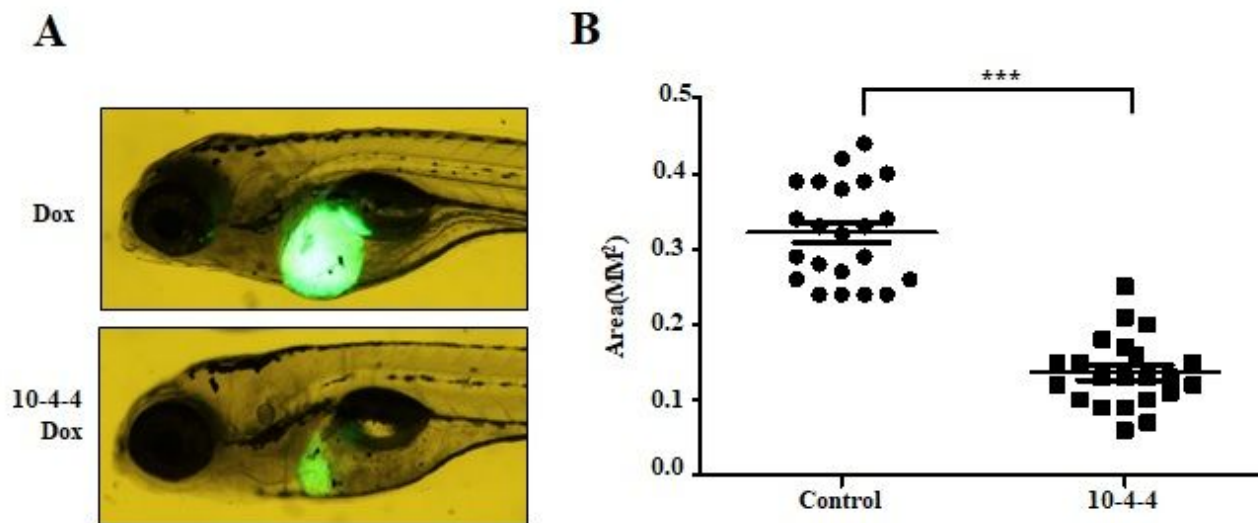


Figure 7

Anti-cancer efficacy of 10-4-4 in vivo. A. 7 dpf *kras*⁺ larvae were treated with 5 μ M 10-4-4 in the presence of 75 μ g/ml Dox. Upper, images of 7 dpf Dox only larvae. Down, representative images of 7 dpf larvae treated with 10-4-4 and Dox. B. 2D liver size was measured based on images. Quantification of liver sizes for *kras*⁺ larvae. N = 22 from each group; *** $p < 0.001$.

Figure 8

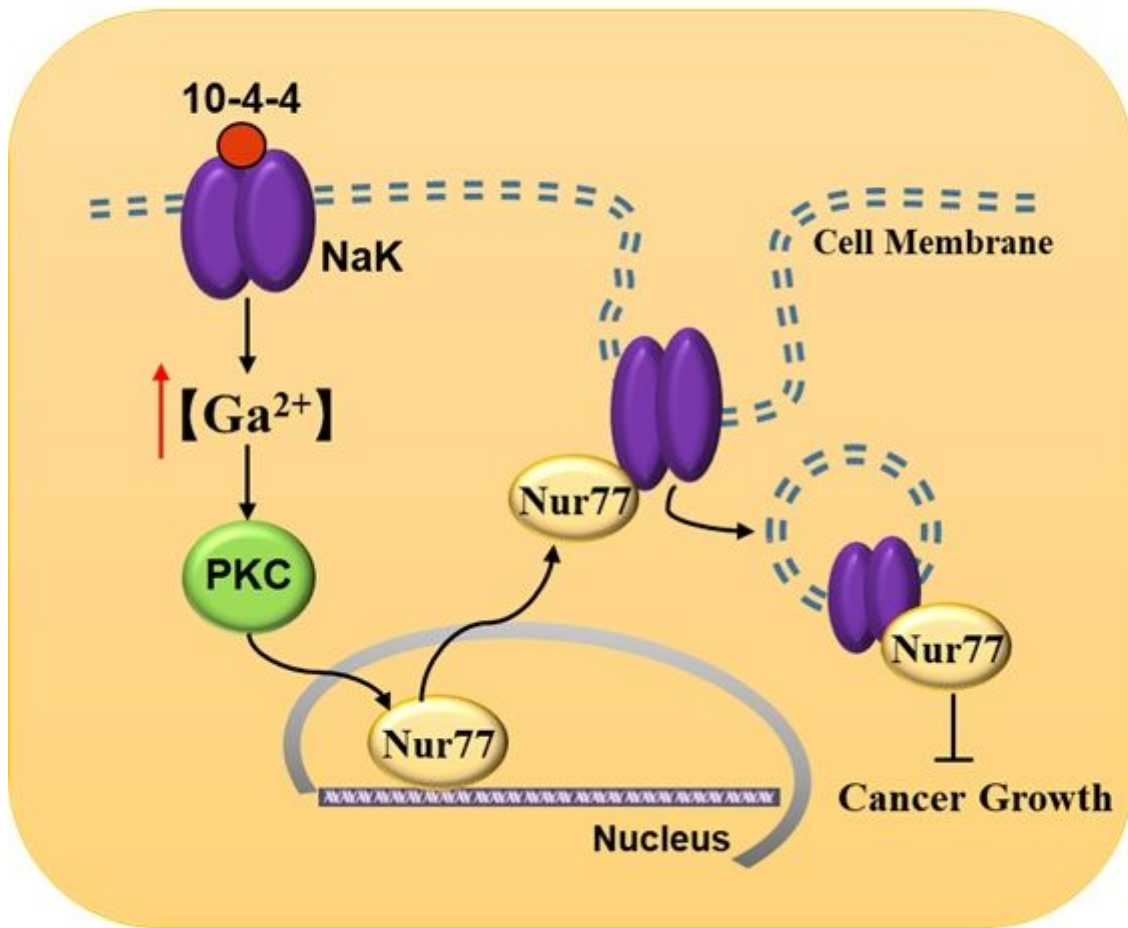


Figure 8

Schematic summary of 10-4-4 mediated signaling pathway.