Bioassay and pathway research of cardiac glycoside from *Streptocaulon juventas*

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Background:

Lung cancer is the most common and lethal disease in the world, but until now, there is no effective agent for lung cancer and most of drugs for lung cancer lack selectivity while having a number of adverse effects.

Over the years, there have been a variety of periodic reports suggesting that digitalis may have an anticancer use. Two decades later, it was observed that breast cancer from women on digitalis had more benign characteristics than cancer cells from control patients not on these drugs, and that five years after a mastectomy, the recurrence among patients not taking digitalis was 9.6 times higher than in patients on these drugs. Surprisingly, these results didn't trigger much research activity at that time to further evaluate a possible benefit for cardiac glycosides in cancer treatment [1].

From 2002 on, Ueda Jun-ya et al. [2-4] published series of reports about the roots of *Streptocaulon juventas* from Vietnamese selectively antiproliferative activity against human lung adenocarcinoma A549 cell line based on the apoptosis, which caused our concentration. *S. juventas,* belonging to Asclepiadaceae family, also produced in Yunnan and Guangxi Province in China. The medicinal part, radix, is used as folk medicine with the action of invigorating the spleen, tonifying and strengthening the kidney [5]. In our study, a fraction from *S. juventas* inhibited the tumor growth of nude mice significantly at day 10 and day 15 during 15-days treatment with the final inhibition rate 58%, more importantly without physical side effects [6]. And then the chemical ingredient from the active fraction was initiated and as a result, 38 cardenolides, including seven new compounds, were isolated and identified using spectroscopic and chemical methods.

In present study, the activity of 34 compounds was tested and we wish to find some interesting compounds which can be candidates for conquering lung cancer.

Materials and Methods:

Antibodies and reagents

The p38 (Thr-180/Tyr-182), PARP-1 and caspase-3 antibodies were purchased from Cell Signaling Technology. Actin (C-11) was obtained from Santa Cruz Biotechnologies. Recombinant human TRIL was obtained from R&D System. Cycloheximide was purchased from Sigma.

Cell culture and cytotoxicity assay

A549 (human lung adenocarcinoma) and Lu-99 (human lung large cell carcinoma) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). These cell lines were maintained as monolayer cultures in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO_2/air at 37°C. PC-9 cell line was maintained in RPMI with both 10% fetal bovine serum (FBS) and L-glutamine (M.A. Bioproducts, Walkersville, MD, USA). HE-Lung cell line was maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum. The quantification of cell viability was performed using the cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) (DOJINDO, Kuma-moto, Japan). A549 cells were plated in 96-well microplates in 10^4 cells/wells and then incubated for 24 h. Sample-containing medium was added into each well, and cells were incubated for another 24 h. As last, 10 μl WST-1 reagent was added into each well, and the absorbance was measured at 450 nm.

Western blotting

A549 cells (3×10^6) were seeded into 6 cm dish and incubated overnight, then treated with sample or verhicle. After incubated for 3, 6, 12 hours, the cells were collected. Briefly, the medium were firstly removed by aspirator, the then remaining cells were washed by previously cooled PBS solution. Then the cells were scraped with 3 mL WCE buffer contained 1 mM DTT, 1 mM PMSF, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate and 0.1 mM EDTA. The cells were vigorously vortexed for 10 s and then centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into a tube and subjected to protein bioassay. All the procedures were in the ice box. Instead of the sample treatment, for positive control, the cells were firstly stimulated by cyclohexiamine, and after 30 min, treated with TRAIL and incubated for 4 hours. The protein content in each sample was adjusted to be the same using WCE buffer and diluted with sample buffer containing DTT, and the heated at 100℃ for 3 min. Cell lysate was transferred to an Immobilon-P-nylon membrane (Millipore). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co. Ltd, Suita, Japan) and probed with primary antibodies. The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and antigoat IgG (DAKO, Glostrup, Denmark) and visualized with the enhanced chemiluminescence system (Amersham Biosciences).

FACS analysis

A549 cells, 3×10^6 for each 6 cm dish, were incubated overnight, and then treated with sample or vehicle. After incubated for 3, 6, 12 hours for samples, the cells were digested with trypsin and suspended with PBS. After centrifuge, the PBS was removed and cells were stained with FITC-conjugated annexin V and PI for 15 min at 20°C in a Ca^{2+}-enriched binding buffer (apoptosis detection kit, R&D Systems, Abingdon, UK; 20). They were immediately analyzed on the flow cytometer in their staining solution.

Result and Discussion

1. The screening of the compound from the 34 compounds using A549 cell line

As shown in Table 1, among the compounds, compound **2**, **10**, **17**, **30**, **31**, **33** showed no activity, compound **1**, **4**, **13**, **25**, **28**, **29**, whose IC_{50} value were over 10μM, showing poor activity. The other compounds displayed stronger activity, especially comp. **16**. In the investigation of the activity at a low concentration 0.1 μM, comp. **8**, **9**, **14**, **16** displayed obviously better activity than other compounds (**Fig. 1**). In order to confirm the effects of

these four compounds on the other cell lines, they were subjected to two kinds of human lung adenocarcinoma (A549 and PC-9), large cell carcinoma (Lu-99) as well as Human lung fibroblast cells (HE-LUNG). As a result, in the range of 0.1 μM- 100 μM, the compounds all dose dependantly inhibited the growth of A549 cells, PC-9 cells and Lu-99 cells. However for HE-LUNG cells, their killing activity were reduced, the cells were about 80% survival after treated by different concentrations of compounds (**Fig. 2**). These meant that the screening compounds were selectively killed the cancer cells but not the normal cells. Considering the simplicity of structure and convenience to be obtained, comp. **9** was selected to perform the mechanism study next.

Table.1 IC_{50} value for every compound

No.	IC_{50}(μM)	No.	IC_{50}(μM)	No.	IC_{50}(μM)
1	36.31	12	4.34	23	2.84
2	-	13	14.75	24	1.26
3	7.01	14	3.14	25	11.67
4	14.02	15	3.22	26	3.25
5	3.32	16	1.26	27	7.95
6	3.64	17	-	28	63.8
7	4.66	18	3.36	29	87.92
8	3.00	19	4.52	30	-
9	2.76	20	3.22	31	-
10	-	21	3.05	32	3.89
11	3.48	22	507.6	33	-
				34	-

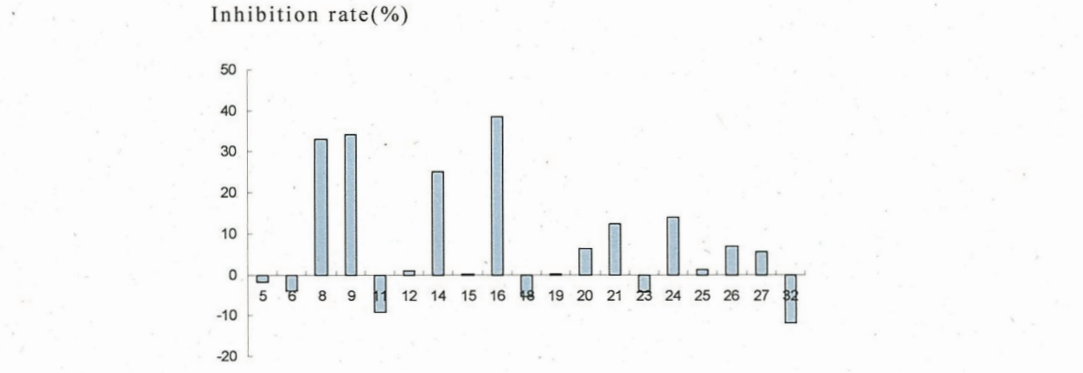


Fig.1 The inhibitory effects of 19 compounds on A549 cells in the concentration of 0.1μM

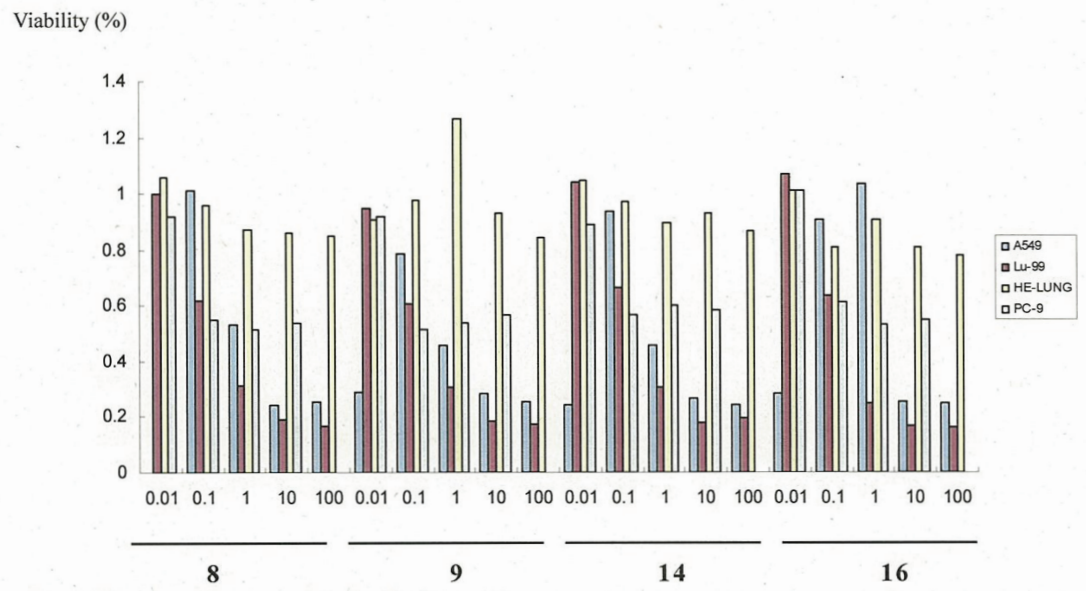


Fig.2 The cytotoxicity of 8, 9, 14, 16 on human lung adenocarcinoma (A549 and PC-9), large cell carcinoma (lu-99), Human lung fibroblast cells (HE-LUNG) (note: activities at 100 μm for PC-9 were not tested.)

2. Flowcytometry analysis of A549 cells treated by compound 9

To confirm the cell death type, we stained the cells with PI and Annexin Ⅴ and analyzed the cell death by FACS. As we know that PI can't penetrate the cell membrane, so it can't stain the cells unless the cells are dead. However, annexin Ⅴ can combined with PS, which is usually located inside of the membrane but turned over when early apoptosis incidence occurred. Thus by double staining, the cell death type can be checked. In this experiment, we investigated the time course at 3hrs, 6hrs and 12hrs. As seen under microscope, the morphology of cells was obviously changed at 12hrs, which is corresponding to the WST-1 result (the inhibition rate was over 40%). By flowcytometric assay, we can find that at 3hrs and 6 hrs, the cells were mostly alive, but at 12hrs, the cells were obviously shifted to the low right area and upright area, which means the cells were happened to apoptosis and necrosis simultaneously (**Fig. 3**).

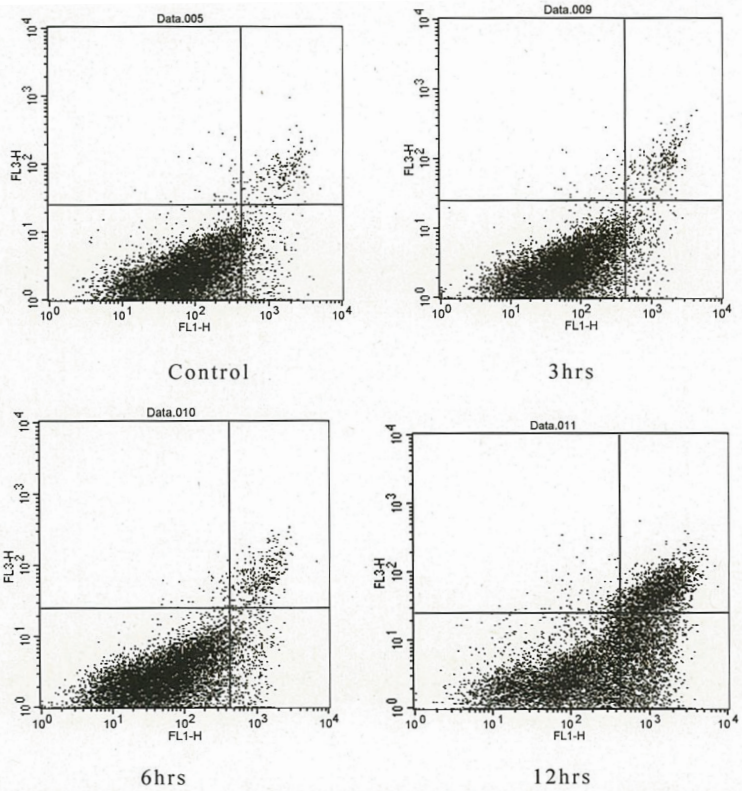


Fig. 3 Flowcytometry analysis of A549 cells treated with comp. 9 at different time

3. Comp.9 induces the cell death in a caspase-independent pathway

As shown in Fig. **4**, it can be seen that in the range of 0.01 to 10μM, Compound **9** could inhibit the growth of A549 cells dose-dependently. And from 0.1 to 10μM, the inhibition rate seemed to be stable and increased slowly. For the time dependant manner, it can be seen that at 12h, the inhibition rate was obviously lower than the other time points. From 24h to 48h, the inhibition rate seemed not to be changed. Under microscope, we can see that the morphology of the treated cells, take 10 μM, 24 h for example, became bigger and round than the normal cells (**Fig. 5**). As we know that cell death has been divided into the apoptosis, autophagy and necrosis, etc [7]. For classic apoptosis, one of the most common characters is that the cells would become smaller. However, in our case, the cells seemed to become bigger, suggesting that the cells maybe not induce the cell death in a classic way.

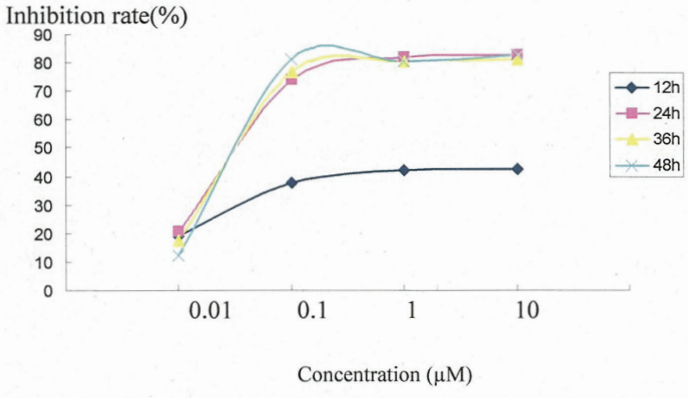
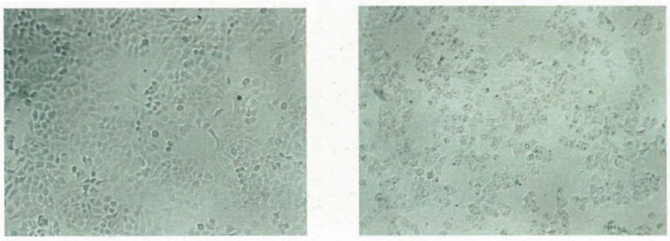


Fig.4 Comp. 9 time and dose dependant manner



Blank Comp.**9** (10μM, 24h)

Fig.5 The morphology of cells treated by comp.9

Apoptosis is dependent upon caspase activation leading to substrate cleavage and, ultimately, cell death. Although required for the apoptotic phenotype, it has become apparent that cells frequently die even when caspase function is blocked. In our experiment, the caspase inhibitor z-VAD-FMK was added into the A549 cells before they were treated with comp. **9**. As a result, the viability was not obviously changed by adding the inhibitor (Fig. 6A), suggesting that the cell death induced by comp.**9** is not dependant on the caspase cascade. On the other hand, TRAIL is a novel apoptosis-inducing antitumor agent, and a clinical evaluation of which is under way. However, due to it's resistant to A549 cells, more and more studies have been performed on the combination treatment with TRAIL and natural compounds to lung cancer. In our study, we found that the combination of comp.**9** and TRAIL can't obviously enhance the apoptosis efficacy. Oppositely, at the dose of 0.1 μM, the viability in the combination group slightly increased compared with TRAIL group (Fig. 6B).

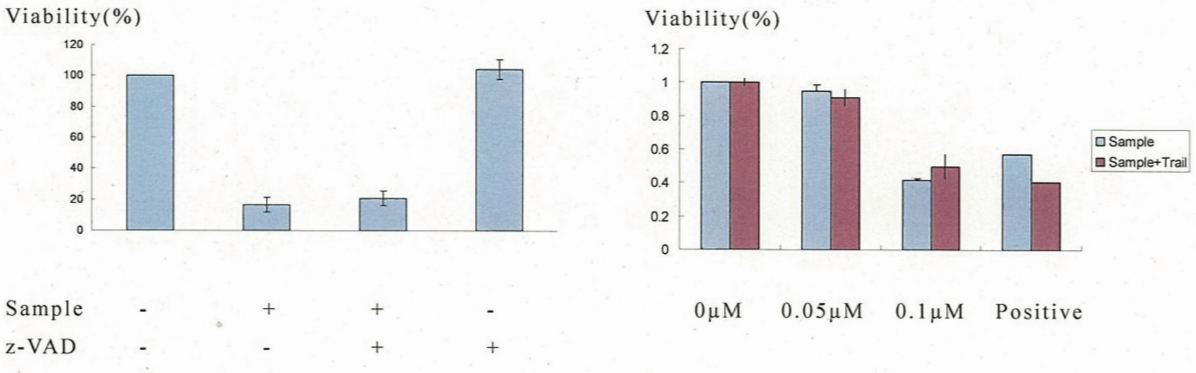


Fig. 6 Effects of comp.9 on A549 cell death. (A: A549 cells were pretreated with 50 μm z-VAD-FMK for 30 min, followed by treatment with sample (1uM) for another 24 hrs, then determined by WST-1 assay. B: A549 cells were pretreated with sample for 30 min, followed by treatment with TRAIL (200ng/mL) for another 24 hrs, then determined by WST-1 assay.)

As mentioned before, the apoptosis was characterized by the caspase cascade and substrate cleavage. In our experiment, the apoptosis markers, caspase-3 and PARP were determined using western blotting. As a result, the cleaved Caspase and Parp cann't be checked in the sample groups at 12hrs, at which we had checked the cell morphology change. However, TRAIL, with the synergy of Cycloheximine, could induce a classic caspase dependant apoptosis. (Fig. 7). All the results support that Comp.**9** can't induce the cell death in a caspase dependant pathway. At least, it's not the primary pathway.

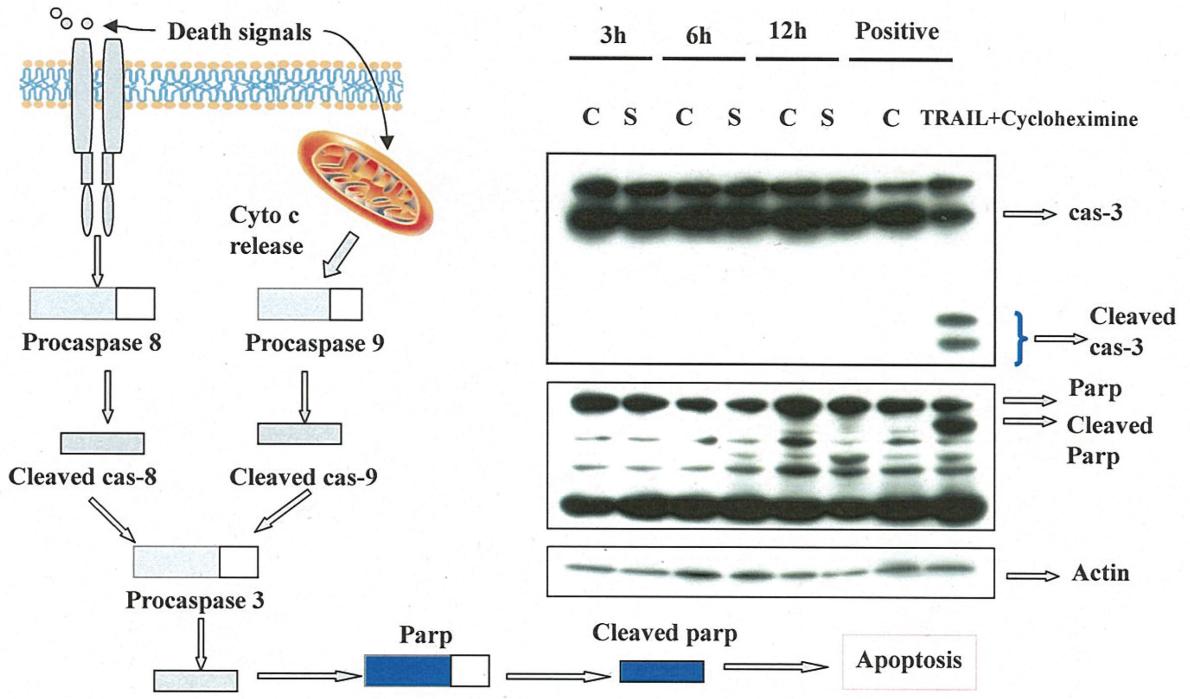


Fig.7 Comp.9 induces the cell death in a caspase-independent pathway (C:control group; S: comp.9 treatment)

4. The cell death was related with SAPK/JNK pathway

Several studies have shown cell death in the absence of caspase activation following death receptor ligation [8]. Here, we use the term necroptosis to describe CICD (caspase-independent cell death) induced by death receptors to distinguish this from MOMP (mitochondrial outer membrane permeabilization)-induced CICD, as the mechanisms of death are almost certainly distinct [9]. Necroptosis induction was reported to be dependent upon RIP-1 and JNK function and appear to occur through macroautophagy (hereafter termed autophagy) [10]. In our experiment, the function of LC-3A/B and pJNK were checked. As shown in **Fig. 8**, the autophagy marker LC-3A/B wasn't obviously changed, but the pJNK, specifically, JNK2/3 and JNK1 was significantly increased from 6 hrs on, suggesting the cell death was related to the SAPK/JNK pathway.

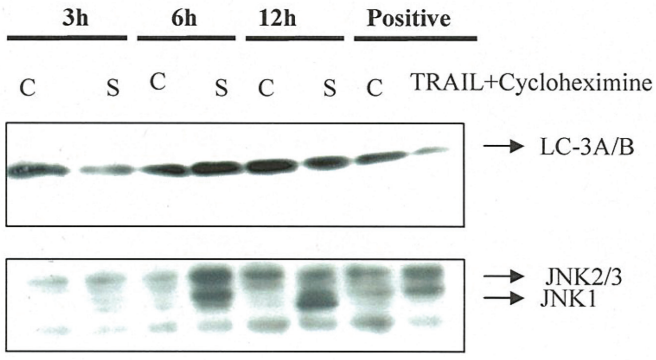


Fig.8 Death receptor-activated necroptosis ((C:control group; S: comp.**9** treatment)

As a cardiac glycoside, comp. **9** may play multiple roles in signal transduction as described [11]. Following cardiac glycoside binding to Na+/K+-ATPase (NKA), the tyrosine kinase SRC is activated and in turn activates the proximal epidermal growth-factor receptor (EGFR). Activated EGFR sequentially recruits the adaptors SHC, growth factor receptor-bound protein 2 (GRB2) and SOS, which ultimately leads to activation of the mitogen-activated protein kinase (MAPK) cascade. In parallel, phospholipase C (PLC) and inositol 1, 4, 5-triphosphate (IP3) also participate in the formation of a functional microdomain that brings NKA into direct contact with the IP3 receptor (IP3R) of the endoplasmic reticulum. At this point, single or repeated transient increases in intracellular Ca2+ are produced. Ca^{2+} oscillations are a universal mode of signaling that mediate a diverse range of cell functions including cell proliferation, differentiation and apoptosis (Fig.9). In our case, comp. **9** obviously adjusted the content of pJNK rather than p38 (data not given), illuminating one of the action target. However, the specific mechanism including the cell death type needs further elucidation.

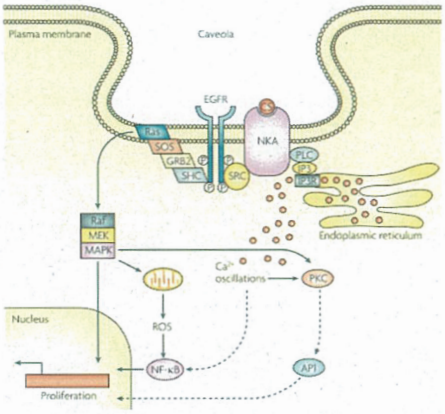


Fig.9 **Na+/K+-ATPase as a versatile signal transducer**

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