Amiloride modulates alternative splicing in leukemic cells and re-sensitizes Bcr-AblT315I mutant cells to imatinib

Wen-Hsin Chang[1], Ta-Chih Liu[1,2,3,8], Wen-Kuang Yang[4], Chien-Chih Lee[5], Yi-Hsiung Lin[5], Tsai-Yun Chen[6], and Jan-Gowth Chang[1,2,3,7,8]

[1]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; [2]Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; [3]Department of Laboratory Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan; [4] Cell/Gene Therapy Research Laboratory, Department of Medical Research, China Medical University and Hospital, Taichung; [5]Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan; [6]Section of Hematology/Oncology, Department of Internal Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan; [7]Cancer Center, Kaohsiung Medical University Hospital; and [8]Center for Excellence in Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.

Running title: To modulate alternative splicing against cancer

Key Words: alternative splicing, amiloride, imatinib

Correspondence

Jan-Gowth Chang, Department of Laboratory Medicine, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan; e-mail: jgchang@ms.kmuh.org.tw

Abstract

The anti-hypertensive drug amiloride is being considered as a tactic to improve cancer therapy including for chronic myelogenous leukemia (CML). In this study, we show that amiloride modulates alternative splicing of various cancer genes, including *Bcl-x*, *HIPK3*, and *BCR/ABL*, and that this effect is not mainly related to pH alteration which is a known effect of the drug. Splice modulation involved various splicing factors themselves, with the phosphorylation state of serine-arginine-rich proteins (SR proteins) also altered during the splicing process. Pretreatment with okadaic acid to inhibit protein phosphatase PP1 reversed partially the phosphorylation levels of SR proteins and also the amiloride-modulated yields of Bcl-x_S and HIPK3 U(-) isoforms. Genome-wide detection of alternative splicing further revealed that many other apoptotic genes were regulated by amiloride, including *APAF-1*, *CRK*, and *SURVIVIN*. Various proteins of the Bcl-2 family and MAPK kinases were found to be involved in amiloride-induced apoptosis. Moreover, the effect of amiloride on mRNA levels of Bcl-x was demonstrated to translate to the protein levels. Cotreatment of K562 and BaF3/Bcr-AblT315I cells with amiloride and imatinib induced more loss of cell viability than either agent alone. Our findings suggest that amiloride may offer a potential treatment option for CML either alone or in combination with imatinib.

Introduction

Alternative splicing (AS), a process that joins different 5' and 3' splice sites of an RNA transcript sequence, is an important post-transcriptional mechanism to generate diverse isoforms of RNAs and encoded proteins from a single primary RNA transcript. Two highly conserved protein families, SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs), are essential factors required for alternative splicing. SR proteins usually bind to splicing enhancers and activate splicing at nearby splice sites. In contrast, hnRNPs usually bind to splicing silencers and antagonize the activity of SR proteins. For example, an excess of SF2/ASF favors the use of proximal 5' splice sites, resulting in exon inclusion, whereas an excess of hnRNP A1 promotes exon skipping by the use of distal 5' splice sites (1). In addition to the relative cellular contents of SR proteins and hnRNPs, reversible phosphorylation status of SR proteins also regulates alternative splicing (2, 3).

Many genes involved in apoptosis have alternative splicing variants with antagonistic function (4). A well-known example is the transcript of bcl-2-related *Bcl-x*, which functions as a dominant regulator of apoptotic cell death by alternative splicing to produce an antiapoptotic Bcl-x_L isoform or a proapoptotic Bcl-x_S isoform (5). Overexpression of Bcl-x_L inhibits apoptosis induced by erythroid differentiation of human leukemic cell lines, supporting its link to poor prognosis in acute myeloid leukemias (6-10), and highly Bcl-x_L expression is associated with increased risk of metastasis and reduced sensitivity to chemotherapeutic treatments in mammary tumors and multiple myeloma (11, 12). In contrast, overexpression of Bcl-x_S sensitizes human breast cancer MCF-7 cells to undergo chemotherapy-induced apoptosis (13), while inhibition of Bcl-x_L expression sensitizes normal human

keratinocytes and epithelial cells to apoptotic stimuli (14). These observations imply that aberrant regulation of alternative splicing of apoptotic genes may be characteristic of human leukemias as well as solid tumors, and manipulation of the alternative splicing of apoptotic genes may have therapeutic potential for cancer therapy.

Amiloride, first approved for clinical use in 1967, is a potassium-sparing diuretic employed in the treatment of hypokalemia, hypertension, and edema (15). In this study, we have uncovered a novel biological action of amiloride, namely modulation of alternative splicing, on human leukemic cells.

Materials and methods

Reagents. Amiloride was purchased from Sigma (St Louis, MO, USA) and was dissolved in DMSO to make 500 mM stock solutions. Imatinib was kindly provided by Novartis Pharmaceuticals and was dissolved in DMSO to make 16 mM stock solutions. Serial dilutions were made in DMSO to obtain final dilutions for cellular assays.

Antibodies. Antibodies were purchased from the following companies: anti-hnRNP C1/C2, phospho-ERK, Bcl-2 and Bax from Santa Cruz (Santa Cruz, CA, USA), anti-PP1, Akt , phospho-PP1 at Thr^{320}, phospho-Akt at Ser^{473} and Thr^{308}, caspase3, and c-Abl from Cell Signaling Technology (Beverly, MA, USA), anti- p38, JNK, phospho-p38, and phospho JNK from BD Biosciences Clontech (Palo Alto, CA, USA), anti-hnRNP A1, hnRNP Q/R, Bcl-x_L, and Bcl-x_S from Sigma (St Louis, MO, USA), anti-actin, histone H3 and caspase9 from Abcam (England), anti-SRp20 and

SF2/ASF from Zymed (San Francisco, CA, USA), anti-A2B1 from Acris (Hiddenhauser, Germany), and anti-ERK from R&D Systems (Minneapolis, MN, USA).

Cell lines. The human leukemia cell lines K562, Molt4, and HL60 were purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan), where each cell line had been tested free of Mycoplasma, bacteria, fungi and cellular contamination. Ba/F3 transfectant (expressing Bcr-Abl with kinase domain point mutations T315I**)** was provided by Michael W. Deininger (16, 17), and confirmed by DNA sequence determination, drug sensitivity test, western blotting analysis, and Mycoplasma test. All cells were maintained in RPMI-1640 medium (Invitrogen Inc, USA) supplemented with 10% fetal bovine serum (Bioind, Kibbutz Beit Haemek, Israel), 2 mM L-glutamine, 1 unit/ml penicillin, and 1 μg/ml streptomycin (Invitrogen Inc, USA) in a humidified 5% CO_2 atmosphere at 37°C. All cell lines were cultured in the laboratory for less than 6 months.

Specimen. Mononuclear cells from four chronic phase and three blast crisis CML patients along with four healthy control individuals were obtained in accordance with an IRB-approved protocol at the Kaohsiung Medical University Hospital.

Cell cycle distribution and apoptosis evaluation. Cells were collected and washed twice with ice-cold PBS. The cell pellet was fixed in 70% ethanol at -20°C overnight and then stained with propidium iodide staining buffer (0.1% TritonX-100, 100 μg/ml RNase A, 500 μg/ml propidium iodide in PBS) for 30min in the dark. Data were collected using a FACScan flow cytometer (Becton Dickinson), and results were

analyzed with CellQuest software (Becton Dickinson).

Cell viability assay. MTT assay was used to determine the viability of treated cells. Briefly, 10 µl MTT (5 mg/ml, Sigma, USA) was added into each culture well after cell treatment for 24 hr with amiloride, imatinib, or both. After 4 hr incubation, the supernatant was removed by centrifugation. DMSO was added and the absorbance at 570 nm was determined by a microplate reader. Each treatment was done at least in triplicate wells and the experiment was repeated three times.

Protein extracts and Western blotting. Cytoplasmic and nuclear fractions of cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Inc., USA) as described previously (18). Total cellular proteins were obtained using cell lysis solution (50 mM Tris-HCl pH 7.5, 137 mM sodium chloride, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 µM leupeptin, and 2 µg/ml aprotinin). Protein samples were separated by SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore). The membrane was blocked with 5% BSA and then exposed to the appropriate concentrations of primary antibodies at 4°C overnight, followed by horseradish peroxidase-conjugated secondary antibody for detection by enzyme chemiluminescence kit (Amersham Inc., USA). Intensity of the signals was measured using LabWorks software (UVP BioImaging Systems).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis. We extracted mRNA from the cells using TurboCapture 8 mRNA kit (Qiagen Inc., USA),

converted it into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. PCR was performed by using specific pairs of primers (Supplementary Table).

RNA extraction and array hybridization. We used the RNeasy Mini kit (Qiagen Inc., USA) to isolate total RNA according to the manufacturer's instructions and verified the RNA quality with a 2100 Bioanalyzer (Agilent). Two μg of total RNA, labeled according to the GeneChip® Whole Transcript Sense Target Labeling Assay manual of the manufacturer (Affymetrix) was hybridized to Human Exon 1.0 ST Arrays (Affymetrix) for 16 hr at 45°C. The hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GCS3000 Scanner (Affymetrix).

Array data analysis. Analysis of variance (ANOVA) p-values and fold changes for gene expression were calculated using Partek Genomic Suit 6.2 (Partek Inc., St. Louis, MO). A threshold cutoff was set to FDR less than 0.01 and at least a 2-fold geometric change in gene-level expression between the untreated versus 0.5 mM amiloride-treated samples. The genes with differential expression were sorted on the basis of gene function using Ingenuity Pathway Analysis software (19). We further selected some candidate genes and performed RT-PCR to verify their altered expression in amiloride-treated cells. The array data have been deposited in Gene Expression Omnibus (GEO; accession number GSE24976).

Statistical analysis. The difference between the amiloride-treated and the control K562 leukemic cells was analyzed by Student's *t*-test with a probability of less than

5% ($P < 0.05$) considered statistically significant.

Results

Amiloride modulates the alternative splicing of *Bcl-x*, *HIPK3*, and *BCR/ABL* fusion genes. With the hypothesis that targeting apoptosis by manipulation of alternative splicing may have therapeutic potential for cancer treatment, we have been searching for small molecules that may regulate the alternative splicing of apoptotic genes in various human cancer cells. Choosing *Bcl-x* and *HIPK3* as the representative for testing various chemicals and drugs, especially those in clinical use, we found that amiloride exerts a potent effect on alternative splicing of these two apoptotic gene transcripts (Fig. 1A and B). As early as 3 hr after the treatment of amiloride, we could detect an increase of pro-apoptotic splice variants, Bcl-x_S and HIPK3 U(-) with a concomitant decrease in the anti-apoptotic splice variants, Bcl-x_L and HIPK3 U(+), which became prominent in 24 hr. Similar splicing patterns were also observed in Molt4 and HL60 cells (Fig. S1). However, the amiloride analogue, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), had no effects on splicing even at doses of equivalent and more potent for Na^+/H^+ channel inhibitory activity. In addition to K562 cells, amiloride also exerted a significant effect in mononuclear cells from chronic phase and blast crisis CML patients (Fig. 1C). These data suggest that amiloride changes the ratio of alternatively spliced isoforms through an enhanced utilization of the upstream alternative 5'-splice site within exon 2 of *Bcl-x* and also increased exon 11 (U) exclusion of *HIPK3*. Interestingly, alternative *BCR/ABL* splice variants were also found in amiloride-treated K562 cells (Fig. 1D). All of these variants were validated by DNA sequencing and further predicted their protein sequence using

ExPASy proteomics tool. Based on the sequencing results, we found that the splicing variant 1 and variant 8 of *BCR/ABL* are already known as b3a2 and e1a2 isoforms. Moreover, the chimeric transcript (b3a2; variant 1) is alternatively spliced into six additional variants (variant 2-7), four of which introduce a premature stop codon. Western blot analysis further confirmed that the expression of BCR/ABL oncoprotein is significantly decreased after the treatment of amiloride.

Amiloride affects the expression levels of SR proteins and hnRNPs.

Demonstrating that amiloride has the ability to modulate pre-mRNA alternative splicing, we then asked whether splicing factors are involved in the process. The results show a significant decrease in the phosphorylation level and total protein level of SF2/ASF in amiloride-treated K562 cells (Fig. 2A). On the contrary, expression of hnRNP A1 was highly expressed after amiloride treatment (Fig. 2B). We also found that hnRNP A2/B1 and hnRNP Q1 were up-regulated, while hnRNP C1/C2, hnRNP I and SRp20 were down-regulated by amiloride (Fig. 2C). In amiloride-treated Molt4 and HL60 cells, we observed a similar decrease in the expression level of SF2/ASF and SRp20 (Fig. S2). These results imply that amiloride may modify the alternative splicing through dephosphorylation of SF2/ASF and also possibly by alterations in the expression levels of various splicing factors.

PP1 but not Akt plays a role in amiloride-induced alternative splicing. Previous studies have shown that SR proteins are among the substrates for Akt kinase and PP1 phosphatase, which may thus act to modify alternative splicing of certain RNA transcripts (20). Our data indicated that amiloride inhibited Akt activity by dephosphorylation of Akt at Ser^{473} and Thr^{308} (Fig 3A), but activated PP1 by

dephosphorylation of Thr^{320} (Fig 3B). Therefore, the decreased level of phosphorylated SR proteins in the cell may result from either inhibition of Akt kinase activity that catalyzes their phosphorylation, or activation of PP1 phosphatase activity that removes the phosphate moieties from SR proteins, or both. With the use of PI3K inhibitors, LY294002 and wortmannin, we found that Akt inactivation was not responsible for dephosphorylation of SF2/ASF or splicing of *Bcl-x* and *HIPK3* (Fig. 3C). To determine whether amiloride-activated PP1 phosphatase plays a role in regulating *Bcl-x* and *HIPK3* alternative splicing, we pre-treated cells with 20 nM okadaic acid to inhibit PP1 phosphatase activity (21) prior to amiloride treatment. The results showed that the okadaic acid pretreatment could relieve partially the effects of amiloride on *Bcl-x* and *HIPK3* splicing in K562 (Fig. 3D), Molt4 and HL60 cells (Fig. S3). This partial relieve implies that other splicing factors such as hnRNPs may also be involved in the splicing process. Pretreatment with okadaic acid could also relieve the effects of amiloride on the dephosphorylation on SF2/ASF and SRp20, but had no definite effect on the expression of hnRNP A1 and hnRNP C1/C2. Taken together, these results suggest that PP1 may in part mediate the effects of amiloride on the alternative splicing of both *Bcl-x* and *HIPK3* through dephosphorylation of SR proteins.

Amiloride affects genome-wide alternative splicing detected by exon arrays.

Since amiloride can modulate the phosphorylation level of SR proteins and the expression level of hnRNPs, we hypothesized that amiloride would have broad effects on pre-mRNA alternative splicing. Analyzing the 17,800 genes represented in "Core" Probeset list, we identified a total of 1288 candidate genes with altered alternative splicing. Gene function analysis indicated that the largest subset (222/1288, 17.2%) of

these candidate genes belongs to the gene expression category, followed by other functional categories such as cell cycle, cellular growth and proliferation, post-translational modification, and cell death. Three more candidates, *APAF-1*, *CRK*, and *SURVIVIN*, of the apoptosis category were selected and validated by RT-PCR (Fig. 4A). Using strict criteria (FDR 0.0001), six more (*MBNL2*, *MIZF*, *PAPD5*, *RFX3*, *SOX6* and *WAC*) were randomly selected from 54 genes to further verify the splicing effects of amiloride. As shown in Figure 4B, distinct alternative splicing patterns were observed after the treatment of amiloride. From the results, it is evident that amiloride has a genome-wide effect on pre-mRNA alternative splicing.

Amiloride induces cell cycle arrest and apoptosis. To evaluate the functional relevance of altering splicing of apoptotic gene transcripts to cancer therapy, we examined the effects of amiloride on cell growth and viability. The results showed a significant increase of K562 cells in S phase of the cell cycle after amiloride treatment (Fig. 5A). Also, addition of 0.5 mM amiloride in the medium resulted in a time-dependent increase in apoptotic sub-G1 phase cells (Fig. 5B). Furthermore, we extended our study to apoptosis-associated molecules and found that amiloride treatment resulted in a significant increase in the active form of caspase 9 and caspase 3 (Fig. 5C), and a dose-dependent cleavage of poly ADP-ribose polymerase (PARP) (Fig. 5D), which are indicative of the induction of apoptosis. To elucidate the signal pathway of amiloride-induced apoptosis, we examined the expression of Bcl-2 family proteins and mitogen-activated protein kinases (MAPKs) by Western blot analysis. The results showed that amiloride downregulated the antiapoptotic proteins Bcl-2 and Bcl-x_L in a dose-dependent manner, while upregulating the proapoptotic proteins Bax and Bcl-x_S (Fig. S4A). As expected, the protein expression levels of Bcl-x_L and

Bcl-x_S were consistent with their RNA expression levels. Finally, we found that amiloride induced a dose-dependent increase in the phosphorylation levels of p38 and JNK, which are pro-apoptotic, with corresponding decrease in the phosphorylation levels of ERK1/2, which enhance cell survival (22, 23), despite unchanged total protein levels of p38, JNK, and ERK in amiloride-treated K562 cells (Fig. S4B). These results indicate that inhibition of ERK, and activation of p38 and JNK may participate in amiloride-induced apoptosis.

Amiloride potentiates the growth inhibitory effect of imatinib. Finally, we sought to see if amiloride can potentiate the growth-inhibition effect of imatinib, the frontline therapy for patients with chronic myeloid leukemia (CML). In imatinib-sensitive K562 cells, we found a dose-dependent decrease in cell viability mediated by imatinib and amiloride individually, and the combination of these two agents produced synergistic effects (Fig. 6A; 6B). Notably, the addition of 0.05 mM amiloride, which does not significantly inhibit the growth of highly imatinib-resistant BaF3/Bcr-AblT315I cells, to a range of concentrations of imatinib, resulted in a significant increase in inhibition of cellular proliferation (Fig. 6C). In addition, the dose-response curve revealed that amiloride concentrations as low as 0.01 mM could increase the growth inhibition effect of 16 μM imatinib (Fig. 6D). However, the combination of imatinib/amiloride did not result in enhanced toxicity toward Bcr-Abl^- HL-60 cells (data not shown). These results indicate that amiloride can not only inhibit the leukemic cell growth, but also synergize with imatinib to Bcr-Abl^+ cells. Moreover, using mononuclear cells from three blast crisis CML patients and four healthy individuals, we found that mononuclear cells' anti-viability response to amiloride is significantly lower in CML patients than in health controls (Fig. S5).

Discussion

Amiloride is an inhibitor of Na^+/H^+ exchanger isoform 1 (NHE1) which is the primary membrane transporter used by cells to regulate intracellular pH (pHi) and cell volume. When cells are stressed by pH changes, splicing site selection in several genes can be altered (24, 25). Our laboratories have previously discovered that an amiloride analogue, EIPA, while reducing pHi, can increase exon 7 inclusion of mutant SMN2 transcript to produce normal SMN protein in spinal muscular atrophy cells (18). Although both amiloride and EIPA are known to decrease pHi values in K562 cells (26), we have found in this study that only amiloride has the ability to modulate alternative splicing, suggesting that the splicing site selection in *Bcl-x* and *HIPK3* by amiloride is mediated through some specific splicing mechanism, rather than pHi change. Previous studies have shown that the ratio of SF2/ASF to hnRNP A1 plays a role in regulating alternative splicing. Similarly, we found amiloride induced exon exclusion of *Bcl-x* and *HIPK3* with concomitant decrease in SF2/ASF and increase in hnRNP A1 expression. Many other splicing factors, such as hnRNP A2/B1, hnRNP C1/C2, hnRNP Q1 and hnRNP I, are also found to be altered by amiloride. Further studies are needed to elucidate how these splicing factors participate in splicing sites selection in amiloride-induced alternative splicing.

Since phosphorylation of SR proteins is important for the pre-mRNA splicing process, we have explored the mechanism of dephosphorylation of SR proteins in cells treated with amiloride. With the use of PI3K and PP1 inhibitors, we found activated PP1 phosphatase rather than inactivated Akt kinase was associated with depophosphorylation of SR proteins, which in turn played a role in amiloride-modified alternative splicing process. In this regard, amiloride appears

similar to ceramide, which regulates the alternative splicing of *Caspase 9* and *Bcl-x* through PP1-mediated splicing mechanism (27). S-adenosylmethionine as well as its metabolites is proapoptotic by stimulating PP1-catalyzed dephosphorylation of RS proteins, resulting in the formation of the alternative variant Bcl-x_S (28). Emetine, an inhibitor of protein synthesis, can also regulate the alternative splicing of *Bcl-x* transcript to produce proapototic Bcl-x_S through a PP1-dependent mechanism (29). Based on our results and these previous studies, we speculate that PP1 may play a key role in modulating the pre-mRNA alternative splicing and that any molecule capable of activating PP1 might have the ability to regulate the alternative splicing of apoptotic gene transcripts. Further studies are needed to verify this hypothesis.

Disturbance of the apoptotic pathway is implicated in many human diseases, especially cancer, and failure to activate this pathway can result in resistance of cancer cells to the cytotoxic effects of multiple chemotherapeutic agents. Therefore, shifting splicing toward the pro-apoptotic isoform offers therapeutic potential in cancer treatment. The splicing of three more apoptosis-related genes, *APAF-1*, *CRK*, and *SURVIVIN*, is found to be altered by amiloride. Apaf-1 functions as an adaptor protein during execution of the mitochondrial pathway of apoptosis. During apoptosis, cytochrome c and dATP can relieve the inhibitory action of the WD-40 repeats and thus enable the oligomerization of Apaf-1 which in turn is able to recruit and activate procaspase-9 (30). Crk oncogene is spliced into two different isoforms, CrkI and CrkII, and only CrkI is found to promote cell migration and invasion by PI3K/Akt signaling (31). Survivin is involved in inhibition of caspases during apoptosis and it also plays critical roles in cell division (32). Although Survivin splice variants have also been correlated with cancer progression, these splice variants are irrelevant to cell proliferation (33). Amiloride also exerts an effect on splicing of the *BCR/ABL*

tyrosine kinase oncogene, which results from a reciprocal translocation of chromosomes 9 and 22 in a hematopoietic stem cell (34). This chimeric oncoprotein, containing a constitutively activated tyrosine kinase domain, plays the key role in malignant transformation and triggers CML (35, 36). Therefore, through modulation of alternative splicing of various apoptotic genes and *BCR/ABL*, amiloride may play a role in producing a proapoptosis phenotype with enhanced sensitization of cells to irradiation or chemotherapeutic drugs, initiating a new target for anticancer therapies.

The tyrosine kinase inhibitor imatinib is an effective, frontline therapy for early, chronic phase CML patients. However, the emergence of imatinib resistance points towards the need to develop novel therapeutic strategies for CML patients. Combining imatinib with other anti-cancer agents is one approach to overcome this problem (37). In this report, we demonstrate for the first time that treatment with a combination of amiloride and imatinib synergistically inhibit the Bcr-Abl$^+$ cell growth. Notably, amiloride could also re-sensitize BaF3/Bcr-AblT315I cells to imatinib. These results provide important in vitro data to support the hypothesis that amiloride could inhibit the Bcr-Abl$^+$ cell growth and proliferation, and may potentially offer a treatment option for CML either alone or in combination with targeted therapy drug such as imatinib. The calculated amiloride concentration in the renal distal tubule is about 3 μM to 20 μM (38). Although the concentration of amiloride used in this study is higher than the published pharmacokinetic data, amiloride still has a significant deleterious effect on T315I cell viability at the concentration of 0.01 mM (Fig. 6D). The concentration of amiloride utilized in this study is higher than that typically achieved when it is used as a potassium-sparing diuretic, suggesting a higher dosage would be needed for use as a chemotherapeutic agent or adjuvant. Teratogenicity and reproduction studies with amiloride in rabbits, mice, and rats given 20 to 25 times the

maximum human dose have been reported (39). These overdose studies revealed no evidence of harm to the fetus or impaired fertility. Moreover, using normal mononuclear cells from healthy individuals, we observed that amiloride did not have a significant effect on cell viability.

In conclusion, we have discovered that amiloride can produce a genome-wide effect on alternative splicing of various RNA transcripts, most importantly including those of the apoptotic factors, in K562 leukemic cells. We also demonstrate that PP1 plays a role in regulating *Bcl-x* and *HIPK3* splicing. In addition, the effects of amiloride on *Bcl-x* splicing are translated to the protein levels and many other apoptotic regulators are found to be involved in amiloride-induced apoptosis (Fig. 7). Moreover, amiloride sensitizes CML cells including the T315I mutation to targeted therapy drug imatinib. These significant findings may have therapeutic potential for cancer treatment by sensitizing CML cells to apoptotic stimuli.

Acknowledgments

This work was supported by the research grant KMU-EM-97-1.1ab-6 from Kaohsiung Medical University, Taiwan (JGC; TCL) and in part by grant DOH96-TD-I-111-TM003 from Department of Health Taiwan (WKY). We thank Dr. Otto O. Yang (UCLA) for reviewing the English writing of this paper.

Authorship

Contribution: Wen-Hsin Chang designed and performed experiments, analyzed data,

and wrote the draft; Chien-Chih Lee, Yi-Hsiung Lin, and Tsai-Yun Chen participated in the coordination of the study; Ta-Chih Liu, Wen- Kuang Yang, and Jan-Gowth Chang conceived/guided the project, and revised the draft.

Reference

1. Mayeda A, Helfman DM, Krainer AR. Modulation of exon skipping and inclusion by heterogeneous nuclear ribonucleoprotein A1 and pre-mRNA splicing factor SF2/ASF. Mol Cell Biol 1993;13:2993-3001.
2. Stamm S. Regulation of alternative splicing by reversible protein phosphorylation. J Biol Chem 2008;283:1223-7.
3. Caceres JF, Kornblihtt AR. Alternative splicing: multiple control mechanisms and involvement in human disease. Trends Genet 2002;18:186-93.
4. Schwerk C, Schulze-Osthoff K. Regulation of apoptosis by alternative pre-mRNA splicing. Mol Cell 2005;19:1-13.
5. Boise LH, Gonzalez-Garcia M, Postema CE, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993;74:597-608.
6. Benito A, Silva M, Grillot D, Nunez G, Fernandez-Luna JL. Apoptosis induced by erythroid differentiation of human leukemia cell lines is inhibited by Bcl-XL. Blood 1996;87:3837-43.
7. Campos L, Rouault JP, Sabido O, et al. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood 1993;81:3091-6.
8. Stoetzer OJ, Nussler V, Darsow M, et al. Association of bcl-2, bax, bcl-xL and interleukin-1 beta-converting enzyme expression with initial response to chemotherapy in acute myeloid leukemia. Leukemia 1996;10 Suppl 3:S18-S22.
9. Karakas T, Maurer U, Weidmann E, Miething CC, Hoelzer D, Bergmann L. High expression of bcl-2 mRNA as a determinant of poor prognosis in acute myeloid leukemia. Ann Oncol 1998;9:159-65.
10. Bincoletto C, Saad ST, da Silva ES, Queiroz ML. Haematopoietic response and bcl-2 expression in patients with acute myeloid leukaemia. Eur J Haematol 1999;62:38-42.
11. Liu R, Page C, Beidler DR, Wicha MS, Nunez G. Overexpression of Bcl-x(L) promotes chemotherapy resistance of mammary tumors in a syngeneic mouse model. Am J Pathol 1999;155:1861-7.

12. Tu Y, Renner S, Xu F, et al. BCL-X expression in multiple myeloma: possible indicator of chemoresistance. Cancer Res 1998;58:256-62.
13. Sumantran VN, Ealovega MW, Nunez G, Clarke MF, Wicha MS. Overexpression of Bcl-XS sensitizes MCF-7 cells to chemotherapy-induced apoptosis. Cancer Res 1995;55:2507-10.
14. Taylor JK, Zhang QQ, Monia BP, Marcusson EG, Dean NM. Inhibition of Bcl-xL expression sensitizes normal human keratinocytes and epithelial cells to apoptotic stimuli. Oncogene 1999;18:4495-504.
15. Bull MB, Laragh JH. Amiloride. A potassium-sparing natriuretic agent. Circulation 1968;37:45-53.
16. O'Hare T, Walters DK, Stoffregen EP, et al. In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. Cancer Res 2005;65:4500-5.
17. La Rosee P, Corbin AS, Stoffregen EP, Deininger MW, Druker BJ. Activity of the Bcr-Abl kinase inhibitor PD180970 against clinically relevant Bcr-Abl isoforms that cause resistance to imatinib mesylate (Gleevec, STI571). Cancer Res 2002;62:7149-53.
18. Yuo CY, Lin HH, Chang YS, Yang WK, Chang JG. 5-(N-ethyl-N-isopropyl)-amiloride enhances SMN2 exon 7 inclusion and protein expression in spinal muscular atrophy cells. Ann Neurol 2008;63:26-34.
19. Cao W, Jamison SF, Garcia-Blanco MA. Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro. RNA 1997;3:1456-67.
20. Blaustein M, Pelisch F, Tanos T, et al. Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. Nat Struct Mol Biol 2005;12:1037-44.
21. Cohen P, Klumpp S, Schelling DL. An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. FEBS Lett 1989;250:596-600.
22. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326-31.
23. Junttila MR, Li SP, Westermarck J. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J 2008;22:954-65.
24. Chen YC, Yuo CY, Yang WK, et al. Extracellular pH change modulates the exon 7 splicing in SMN2 mRNA. Mol Cell Neurosci 2008;39:268-72.
25. Borsi L, Balza E, Gaggero B, Allemanni G, Zardi L. The alternative splicing pattern of the tenascin-C pre-mRNA is controlled by the extracellular pH. J Biol Chem 1995;270:6243-5.

26. He B, Deng C, Zhang M, Zou D, Xu M. Reduction of intracellular pH inhibits the expression of VEGF in K562 cells after targeted inhibition of the Na+/H+ exchanger. Leuk Res 2007;31:507-14.
27. Chalfant CE, Rathman K, Pinkerman RL, et al. De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1. J Biol Chem 2002;277:12587-95.
28. Yang H, Sadda MR, Li M, et al. S-adenosylmethionine and its metabolite induce apoptosis in HepG2 cells: Role of protein phosphatase 1 and Bcl-x(S). Hepatology 2004;40:221-31.
29. Boon-Unge K, Yu Q, Zou T, Zhou A, Govitrapong P, Zhou J. Emetine regulates the alternative splicing of Bcl-x through a protein phosphatase 1-dependent mechanism. Chem Biol 2007;14:1386-92.
30. Lauber K, Appel HA, Schlosser SF, Gregor M, Schulze-Osthoff K, Wesselborg S. The adapter protein apoptotic protease-activating factor-1 (Apaf-1) is proteolytically processed during apoptosis. J Biol Chem 2001;276:29772-81.
31. Takino T, Nakada M, Miyamori H, Yamashita J, Yamada KM, Sato H. CrkI adapter protein modulates cell migration and invasion in glioblastoma. Cancer Res 2003;63:2335-7.
32. Altieri DC. Validating survivin as a cancer therapeutic target. Nat Rev Cancer 2003;3:46-54.
33. Noton EA, Colnaghi R, Tate S, et al. Molecular analysis of survivin isoforms: evidence that alternatively spliced variants do not play a role in mitosis. J Biol Chem 2006;281:1286-95.
34. Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. Blood 2000;96:3343-56.
35. Brauer KM, Werth D, von Schwarzenberg K, et al. BCR-ABL activity is critical for the immunogenicity of chronic myelogenous leukemia cells. Cancer Res 2007;67:5489-97.
36. Arana-Trejo RM, Ruiz Sanchez E, Ignacio-Ibarra G, et al. BCR/ABL p210, p190 and p230 fusion genes in 250 Mexican patients with chronic myeloid leukaemia (CML). Clin Lab Haematol 2002;24:145-50.
37. Kano Y, Akutsu M, Tsunoda S, et al. In vitro cytotoxic effects of a tyrosine kinase inhibitor STI571 in combination with commonly used antileukemic agents. Blood 2001;97:1999-2007.
38. Smith AJ, Smith RN. Kinetics and bioavailability of two formulations of amiloride in man. Br J Pharmacol 1973;48:646-9.
39. Qasqas SA, McPherson C, Frishman WH, Elkayam U. Cardiovascular pharmacotherapeutic considerations during pregnancy and lactation. Cardiol Rev

2004;12:240-61.

Figure legends

Figure 1. Effects of amiloride on alternative splicing of *Bcl-x*, *HIPK3*, and *BCR/ABL RNAs*. Messenger RNA was extracted and detected by RT-PCR for alternative splicing of *Bcl-x, HIPK3,* or *BCR/ABL.* The splicing variants and their expected PCR products using the primers indicated by arrowheads are illustrated on the left column. (A, B) K562 cells were treated with various concentrations of amiloride or EIPA (middle column) for 24 h, or treated with 0.5 mM amiloride for 3, 6, 12, and 24 h (right column). (C) Mononuclear cells from CML patients, four at chronic phase and three during blastic crisis, were treated without (-) or with (+) 0.3 mM amiloride for 24 h. Results of *Bcl-*x_S/x_L and *HIPK*3 U(-)/U(+) ratios of the amiloride-treated (+) are normalized to those of untreated condition (-). (D) RT-PCR and western blot results from k562 cells treated without or with 0.5 mM amiloride for 24 h. Aliquots containing 20 μg of whole cell lysates were separated by SDS-PAGE and immunoblotted with antibodies against c-abl. Actin was shown as internal standards.

Figure 2. Effects of amiloride on SR proteins and hnRNPs. Cells were treated with amiloride and then harvested. Equal amounts of nuclear extracts (A and C: 10 ug) or whole cell lysates (B: 20 ug) were separated by SDS-PAGE and immunoblotted with various antibodies as indicated. Histone H3 and Actin were shown as internal standards.

Figure 3. Effects of Akt kinase and PP1 phosphatase on amiloride-induced alternative splicing. Cells were treated with different concentrations of amiloride for 24h and then harvested. Aliquots containing 20 ug of cytoplasmic and 10 ug of

nuclear extracts (A), or 20 ug of whole cell lysates (B) were subjected to SDS-PAGE followed by immunoblot analysis using specific antibodies. Cells were treated with amiloride, LY294002, and Wortmannin for 24h respectively (C), or treated with okadaic acid for 30 min and then exposed to amiloride for 6 hr (D). The effects of amiloride and inhibitors on alternative splicing of *Bcl-x* and *HIPK3* were detected by RT-PCR. Equal amounts of nuclear extracts (10 ug) were separated by SDS-PAGE and immunoblotted with various antibodies as indicated. Histone H3 and actin were used as internal standards.

Figure 4. RT-PCR validation of candidate alternative splicing genes. Cells were treated with various doses of amiloride (A), or with 0.5 mM amiloride (B) for 24 h. Messenger RNA was extracted and detected by RT-PCR for the alternative splicing of various candidate genes as indicated.

Figure 5. Effects of amiloride on cell cycle and apoptosis. (A) Cell cycle distribution determined by flow cytometry. (B) Sub-G1population obtained from the cells of flow cytometry data of propidium iodide fluorescence. (C) Cells were treated for 24h with the indicated concentration of amiloride. Aliquots containing 20 ug cytoplasmic extracts, or (D) 10 ug of nuclear extracts were separated by SDS-PAGE and immunoblotted with indicated antibodies. Histone H3 and actin were used as internal standards.

Figure 6. Treatment with amiloride enhances sensitization of CML cells to imatinib. K562 cells (A; B), and BaF3/Bcr-AblT315I cells (C; D) were treated with amiloride, imatinib, or both. Cell viability was assayed by MTT. All values represent

the means ± SD of triplicates performed on 3 separate occasions.

Figure 7. Hypothetical schematic diagram of the amiloride-induced alternative splicing in K562 cells.

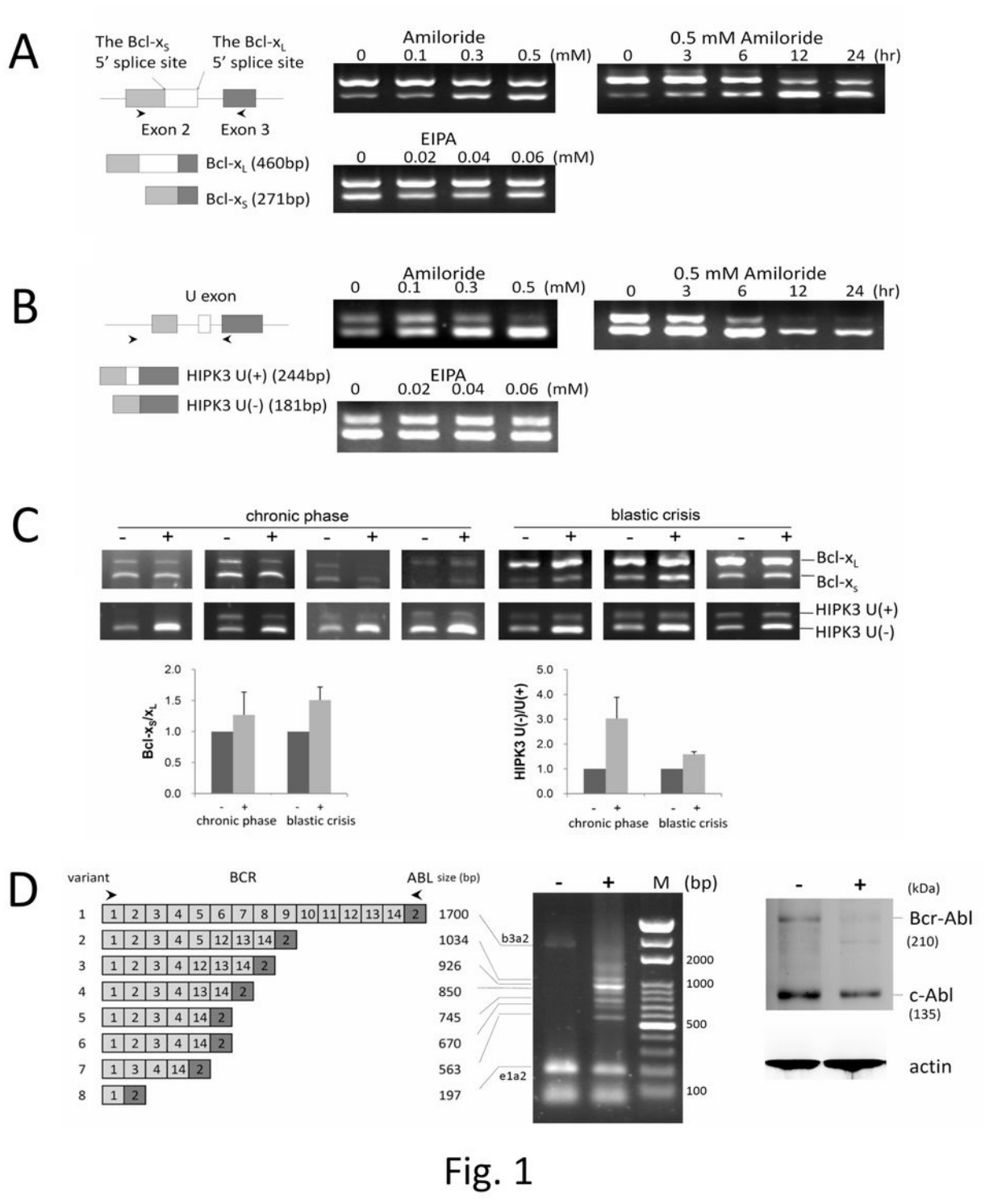

A
The Bcl-xS 5′ splice site
The Bcl-xL 5′ splice site
Exon 2
Exon 3
Bcl-xL (460bp)
Bcl-xS (271bp)
Amiloride
0 0.1 0.3 0.5 (mM)
0.5 mM Amiloride
0 3 6 12 24 (hr)
EIPA
0 0.02 0.04 0.06 (mM)
B
U exon
HIPK3 U(+) (244bp)
HIPK3 U(-) (181bp)
Amiloride
0 0.1 0.3 0.5 (mM)
0.5 mM Amiloride
0 3 6 12 24 (hr)
EIPA
0 0.02 0.04 0.06 (mM)
C
chronic phase
blastic crisis
- + - + - + - + - + - + - + - +
Bcl-xL
Bcl-xS
HIPK3 U(+)
HIPK3 U(-)
Bcl-xS/xL
2.0
1.5
1.0
0.5
0.0
- + - +
chronic phase blastic crisis
HIPK3 U(-)/U(+)
5.0
4.0
3.0
2.0
1.0
0.0
- + - +
chronic phase blastic crisis
D
variant
BCR
ABL
size (bp)
1 1 2 3 4 5 6 7 8 9 10 11 12 13 14 2
2 1 2 3 4 5 12 13 14 2
3 1 2 3 4 12 13 14 2
4 1 2 3 4 13 14 2
5 1 2 3 4 14 2
6 1 2 3 4 14 2
7 1 3 4 14 2
8 1 2
1700
1034
926
850
745
670
563
197
b3a2
e1a2
- + M (bp)
2000
1000
500
100
- + (kDa)
Bcr-Abl
(210)
c-Abl
(135)
actin

Fig. 1

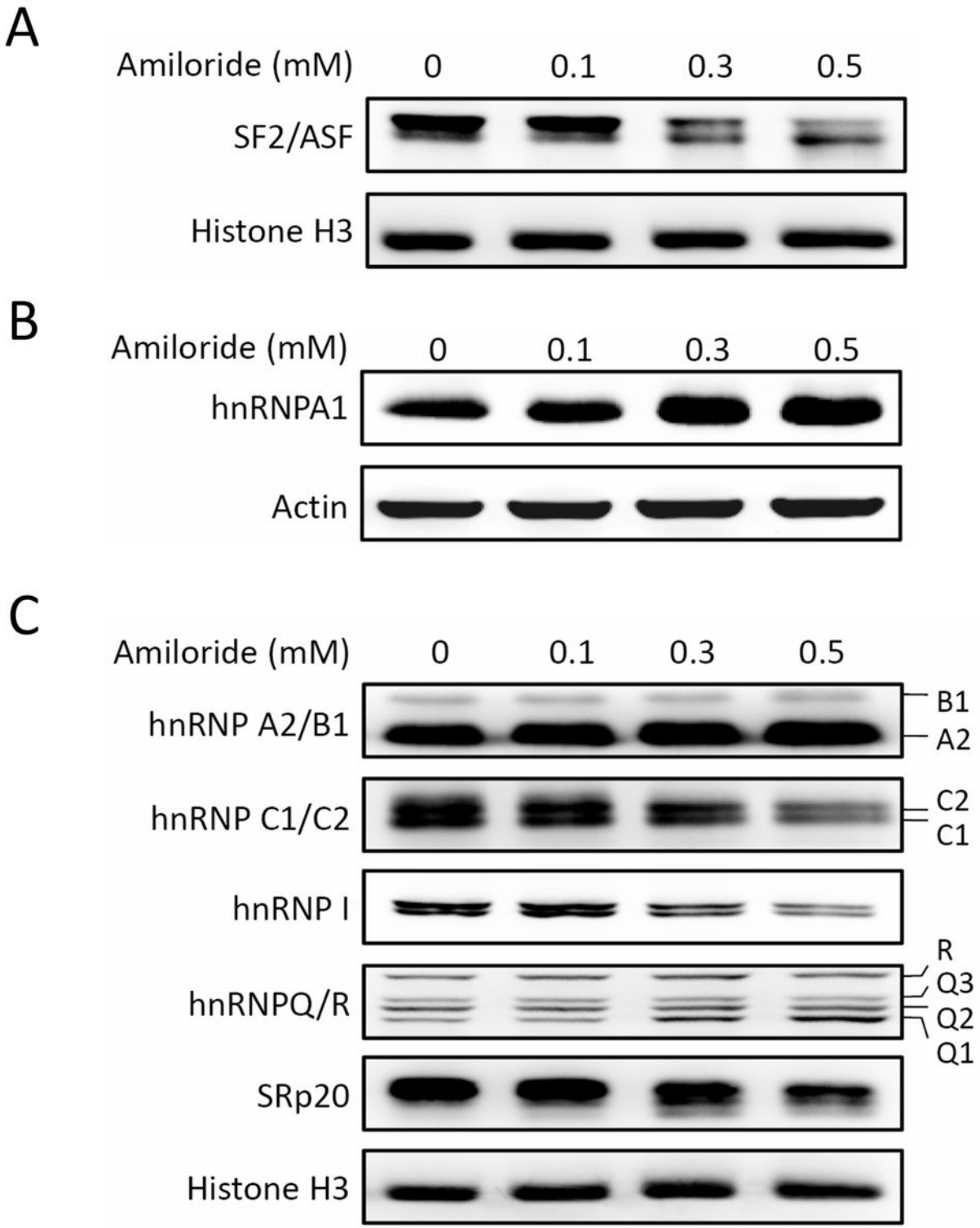

A
Amiloride (mM)
0
0.1
0.3
0.5
SF2/ASF
Histone H3
B
Amiloride (mM)
0
0.1
0.3
0.5
hnRNPA1
Actin
C
Amiloride (mM)
0
0.1
0.3
0.5
hnRNP A2/B1
B1
A2
hnRNP C1/C2
C2
C1
hnRNP I
hnRNPQ/R
R
Q3
Q2
Q1
SRp20
Histone H3

Fig. 2

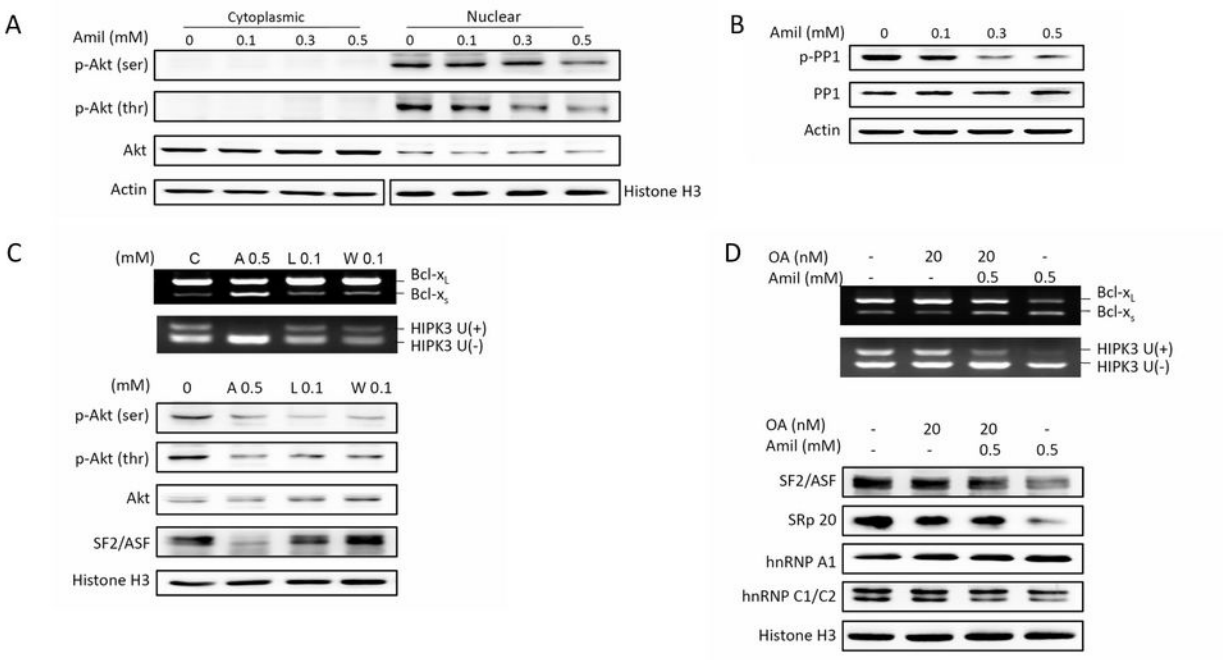
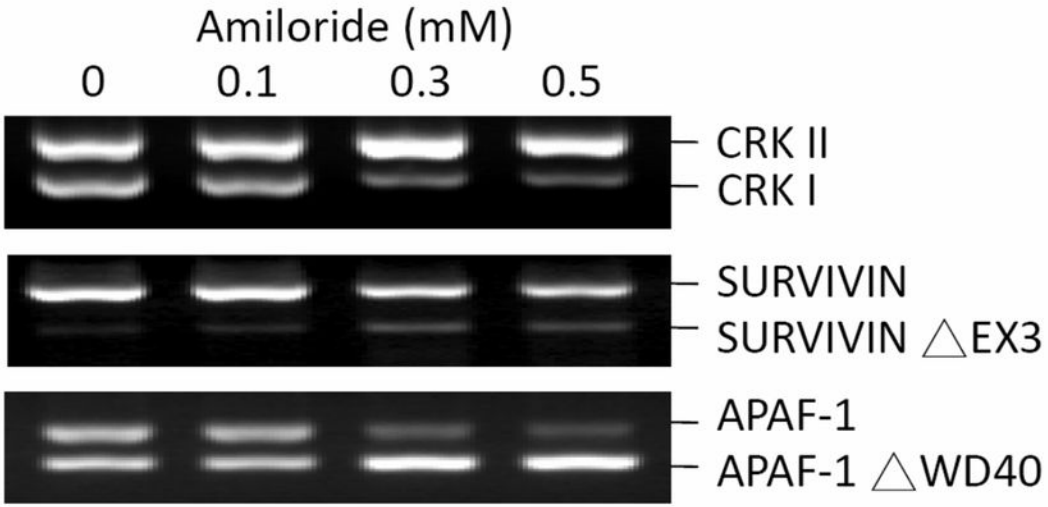

A
Cytoplasmic
Nuclear
Amil (mM)
0
0.1
0.3
0.5
0
0.1
0.3
0.5
p-Akt (ser)
p-Akt (thr)
Akt
Actin
Histone H3
B
Amil (mM)
0
0.1
0.3
0.5
p-PP1
PP1
Actin
C
(mM)
C
A 0.5
L 0.1
W 0.1
Bcl-x$_L$
Bcl-x$_s$
HIPK3 U(+)
HIPK3 U(-)
(mM)
0
A 0.5
L 0.1
W 0.1
p-Akt (ser)
p-Akt (thr)
Akt
SF2/ASF
Histone H3
D
OA (nM)
-
20
20
-
Amil (mM)
-
-
0.5
0.5
Bcl-x$_L$
Bcl-x$_s$
HIPK3 U(+)
HIPK3 U(-)
OA (nM)
-
20
20
-
Amil (mM)
-
-
0.5
0.5
SF2/ASF
SRp 20
hnRNP A1
hnRNP C1/C2
Histone H3

Fig. 3

A


Amiloride (mM)
0
0.1
0.3
0.5
CRK II
CRK I
SURVIVIN
SURVIVIN △EX3
APAF-1
APAF-1 △WD40

B

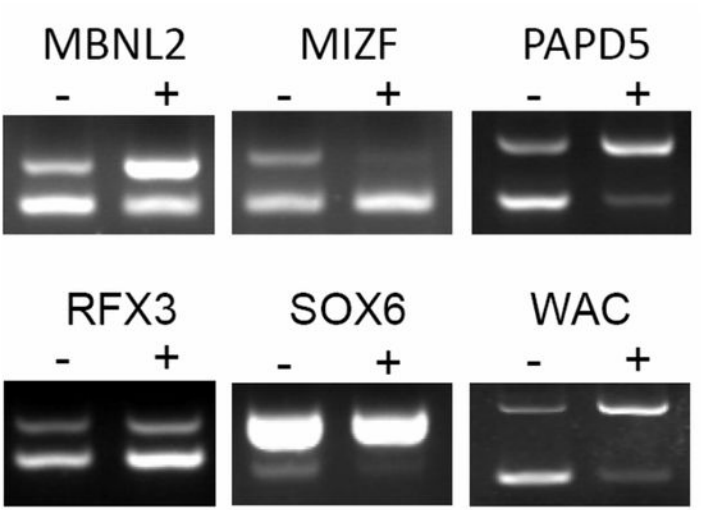

MBNL2
MIZF
PAPD5
RFX3
SOX6
WAC
-
+

Fig. 4

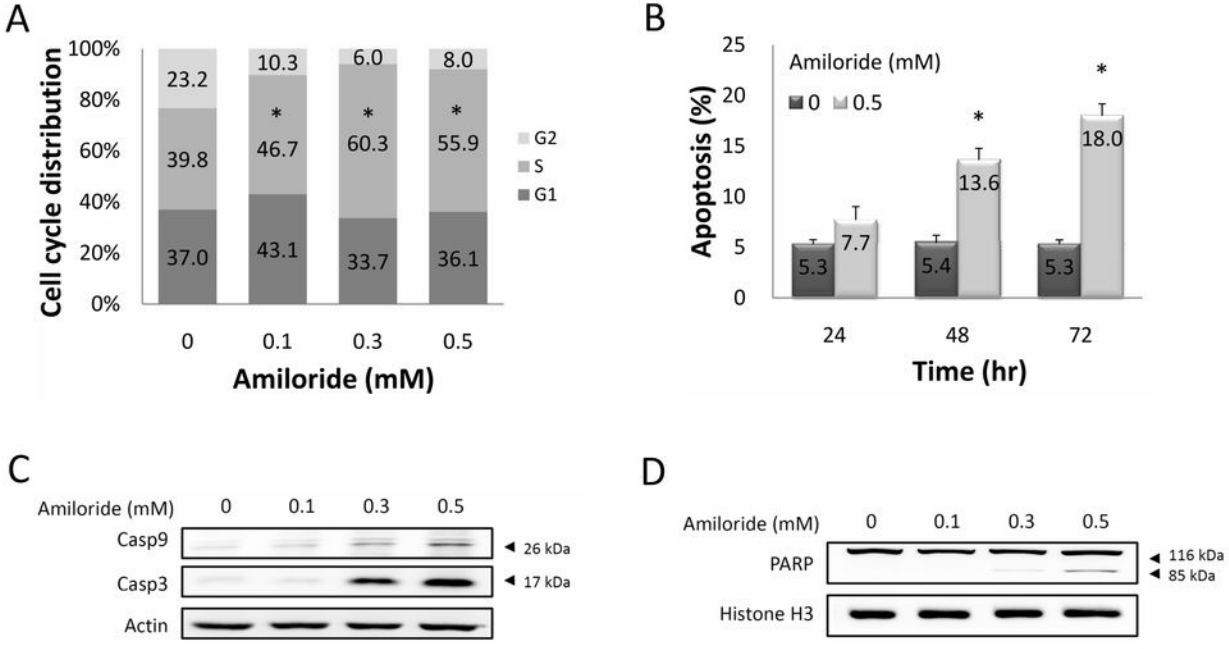

A
Cell cycle distribution
100%
80%
60%
40%
20%
0%
23.2
39.8
37.0
10.3
46.7
43.1
6.0
60.3
33.7
8.0
55.9
36.1
*
*
*
G2
S
G1
0
0.1
0.3
0.5
Amiloride (mM)
B
Apoptosis (%)
25
20
15
10
5
0
Amiloride (mM)
0
0.5
5.3
7.7
5.4
13.6
5.3
18.0
*
*
24
48
72
Time (hr)
C
Amiloride (mM)
0
0.1
0.3
0.5
Casp9
Casp3
Actin
26 kDa
17 kDa
D
Amiloride (mM)
0
0.1
0.3
0.5
PARP
Histone H3
116 kDa
85 kDa

Fig. 5

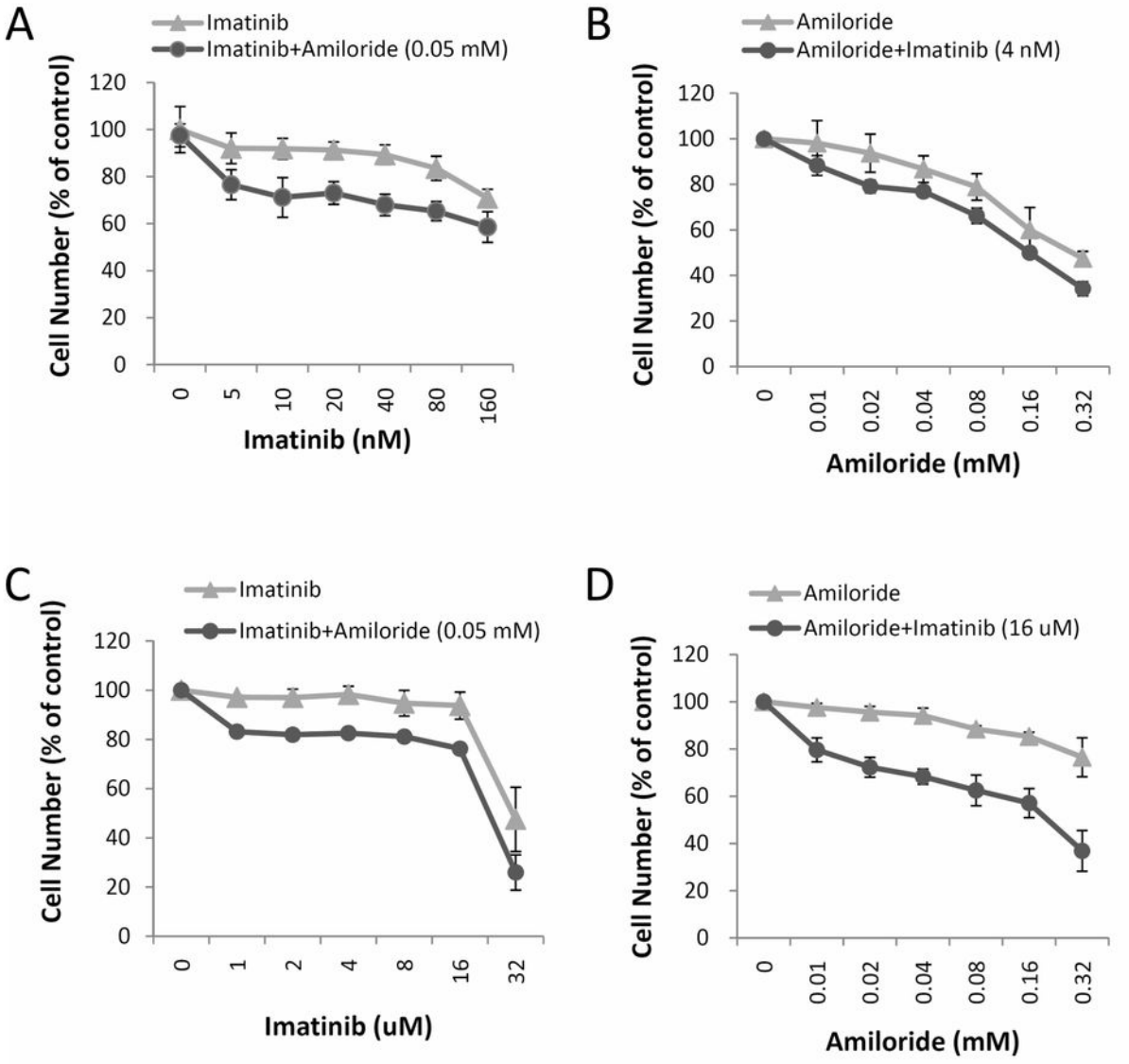

A
Imatinib
Imatinib+Amiloride (0.05 mM)
Cell Number (% of control)
120
100
80
60
40
20
0
0
5
10
20
40
80
160
Imatinib (nM)
B
Amiloride
Amiloride+Imatinib (4 nM)
Cell Number (% of control)
120
100
80
60
40
20
0
0
0.01
0.02
0.04
0.08
0.16
0.32
Amiloride (mM)
C
Imatinib
Imatinib+Amiloride (0.05 mM)
Cell Number (% of control)
120
100
80
60
40
20
0
0
1
2
4
8
16
32
Imatinib (uM)
D
Amiloride
Amiloride+Imatinib (16 uM)
Cell Number (% of control)
120
100
80
60
40
20
0
0
0.01
0.02
0.04
0.08
0.16
0.32
Amiloride (mM)

Fig. 6

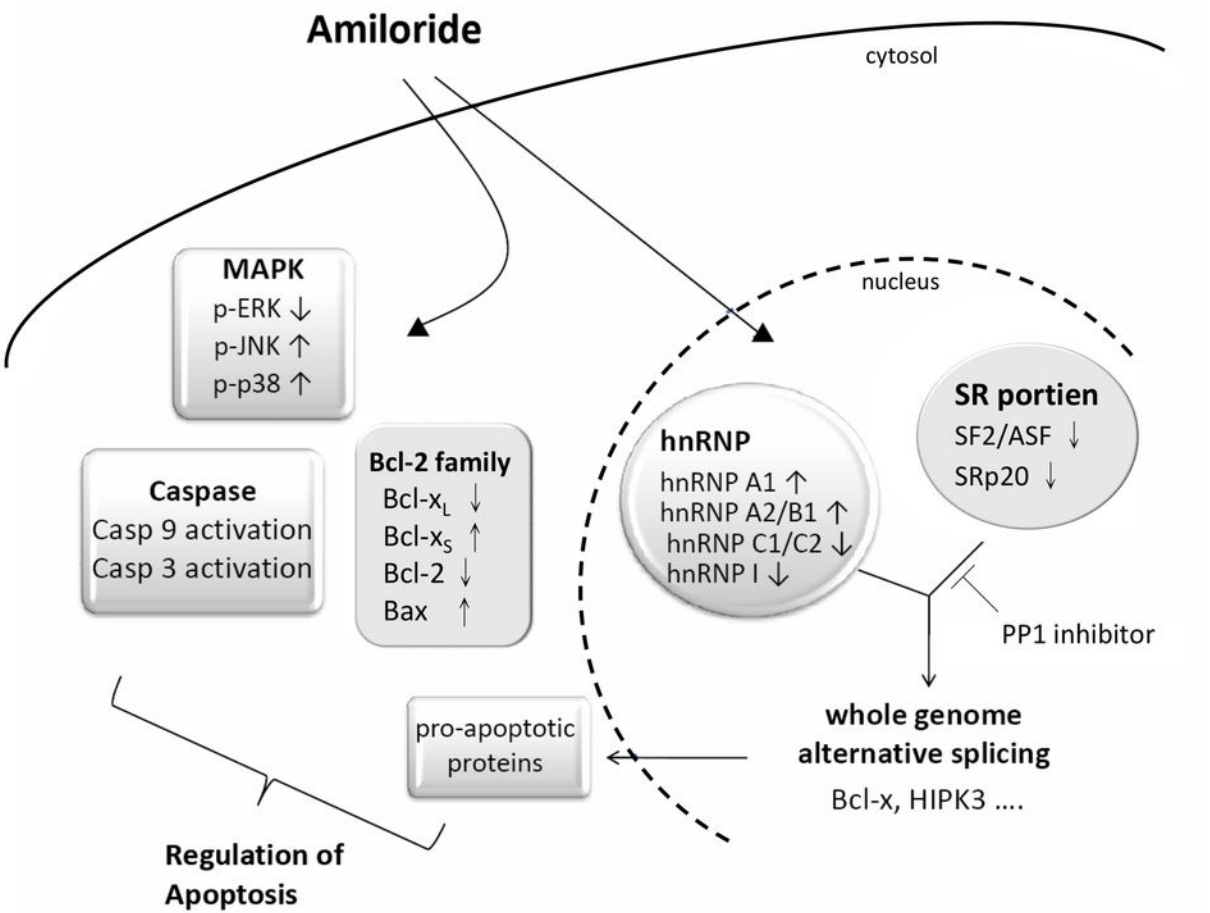

Amiloride
cytosol
MAPK
p-ERK ↓
p-JNK ↑
p-p38 ↑
nucleus
SR portien
SF2/ASF ↓
SRp20 ↓
hnRNP
hnRNP A1 ↑
hnRNP A2/B1 ↑
hnRNP C1/C2 ↓
hnRNP I ↓
PP1 inhibitor
Caspase
Casp 9 activation
Casp 3 activation
Bcl-2 family
Bcl-x$_L$ ↓
Bcl-x$_S$ ↑
Bcl-2 ↓
Bax ↑
pro-apoptotic proteins
whole genome alternative splicing
Bcl-x, HIPK3 ....
Regulation of Apoptosis

Fig. 7