Amiloride Modulates Alternative Splicing in Leukemic Cells and Resensitizes Bcr-AbiT315I Mutant Cells to Imatinib

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Abstract

The antihypertensive drug amiloride is being considered as a tactic to improve cancer therapy including that for chronic myelogenous leukemia. In this study, we show that amiloride modulates the 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, with the phosphorylation state of serine-arginine–rich (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-xL 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-AbiT315I 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 chronic myelogenous leukemia either alone or in combination with imatinib.

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Introduction

Alternative splicing (AS), a process that joins different 5’ and 3’ splice sites of an RNA transcript sequence, is an important posttranscriptional mechanism to generate diverse isoforms of RNAs and encoded proteins from a single primary RNA transcript. Two highly conserved protein families, serine-arginine–rich (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, the 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-xL isoform or a proapoptotic Bcl-xS isoform (5). Overexpression of Bcl-xL 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-xL expression is associated with an increased risk of metastasis and reduced sensitivity to chemotherapeutic treatments in mammary tumors and multiple myeloma (11, 12). In contrast, overexpression of Bcl-xS sensitizes human breast cancer MCF-7 cells to undergo chemotherapy-induced apoptosis (13), while inhibition of Bcl-xL expression sensitizes normal human keratinocytes and epithelial cells to apoptotic stimuli (14). These observations imply that aberrant regulation of the 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 and was dissolved in DMSO to make 500-mmol/L stock solutions. Imatinib was kindly provided by Novartis Pharmaceuticals and was dissolved in DMSO to make 16-mmol/L 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 Biotechnology); anti-PP1, Akt, phospho-PP1 at Thr308, phospho-Akt at Ser473 and Thr308, caspase 3, and c-Abl from Cell Signaling Technology; anti-p38, JNK, phospho-cell line was fixed in 70% ethanol at

Cell cycle distribution and apoptosis evaluation

Cell viability assay

MTT assay was used to determine the viability of treated cells. Briefly, 10 μL MTT (5 mg/mL) was added into each culture well after cell treatment for 24 hours with amiloride, imatinib, or both. After 4 hours of 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 3 times.

Protein extracts and Western blotting

Cytoplasmic and nuclear fractions of cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Inc.) as described previously (18). Total cellular proteins were obtained using cell lysis solution (50 mmol/L Tris-HCl, pH 7.5, 137 mmol/L sodium chloride, 1 mmol/L EDTA, 1% Nonidet P-40, 10% glycerol, 0.1 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 20 mmol/L β-glycerophosphate, 50 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μmol/L 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.). The intensity of the signals was measured using LabWorks software (UVP Bioimaging Systems).

Reverse transcription PCR analysis

We extracted mRNA from the cells using TurboCapture 8 mRNA kit (Qiagen Inc.), converted it into cDNA using M-MLV reverse transcriptase (Promega) 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.) to isolate total RNA according to the manufacturer’s instructions and verified the RNA quality with a 2100 Bioanalyzer (Agilent). Two nanograms 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 hours 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) F-values and fold changes for gene expression were calculated using Partek Genomic Suit 6.2 (Partek Inc.). A threshold cutoff was set to FDR < 0.01 and at least a 2-fold geometric change in gene-level expression between the untreated versus the 0.5-mmol/L 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 the 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, particularly those in clinical use, we found that amiloride exerts a potent effect on the alternative splicing of these 2 apoptotic gene transcripts (Fig. 1A and B). As early as 3 hours after the treatment of amiloride, we could detect an increase of proapoptotic splice variants, Bcl-xS and HIPK3 U(-), with a concomitant decrease in the antiapoptotic splice variants, Bcl-xL and HIPK3 U(+), which became prominent in 24 hours. Similar splicing patterns were also observed in Molt4 and HL60 cells (Supplementary 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 substantial 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 Bcl-x. 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 we further predicted their protein sequence with the ExPASy proteomics tool. Based on the sequencing results, we found that the splicing variants 1 and 8 of BCR/ABL are already known as b3a2 and e1a2 isoforms. Moreover, the chimeric transcript (b3a2; variant 1) is alternatively spliced into 6 additional variants (variants 2–7), 4 of which introduce a premature stop codon. Western blot analysis further confirmed that the expression of BCR/ABL oncoprotein is substantially decreased after the treatment of amiloride.

Amiloride affects the expression levels of serine-arginine–rich 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 considerable decrease in the phosphorylation level and total protein level of SF2/ASF in amiloride-treated K562 cells (Fig. 2A). On the contrary, the expression of hnRNP A1 was highly expressed after amiloride treatment (Fig. 2B). We also found that hnRNP A2/B1 and hnRNP Q1 were upregulated, whereas hnRNP C1/C2, hnRNP I, and Srp20 were downregulated by amiloride (Fig. 2C). In amiloride-treated MolH and HL60 cells, we observed a similar decrease in the expression level of SF2/ASF and Srp20 (Supplementary 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\(^{173}\) and Thr\(^{308}\) (Fig 3A) but activated PP1 by the dephosphorylation of Thr\(^{230}\) (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 the activation of PP1 phosphatase activity that removes the phosphate moieties from SR proteins, or both. With the use of P38 inhibitors, LY294002, and wortmannin, we found that Akt inactivation was not responsible for the dephosphorylation of SF2/ASF or the splicing of Bcl-x and HIPK3 (Fig. 3C). To find whether amiloride-activated PP1 phosphatase plays a role in regulating Bcl-x and HIPK3 alternative splicing, we pretreated cells with 20 nmol/L okadaic acid to inhibit PP1 phosphatase activity (21) prior to amiloride treatment. The results showed that the okadaic acid pretreatment could partially relieve the effects of amiloride on Bcl-x and HIPK3 alternative splicing, where hnRNP A2/B1 and hnRNP Q1 were upregulated, whereas hnRNP C1/C2, hnRNP I, and SRp20 were downregulated (Supplementary Fig. S3). This partial relief 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 of 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 the 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 the "Core" Probeset list, we identified a total of 1,288 candidate genes with altered alternative splicing. Gene function analysis indicated that the largest subset (222/1,288, 17.2%) of these genes with altered alternative splicing. Analyzing the 17,800 genes represented in the "Core" Probeset list, we identified a total of 1,288 candidate genes with altered alternative splicing. Gene function analysis indicated that the largest subset (222/1,288, 17.2%) of these gene expression category, followed by other functional categories such as cell cycle, cellular growth and proliferation, posttranslational 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), 6 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 with 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 the splicing of apoptotic gene transcripts to cancer therapy, we examined the effects of amiloride on cell growth and viability. The results showed a substantial increase in K562 cells in the S phase of the cell cycle after amiloride treatment (Fig. 5A). Also, the addition of 0.5 mmol/L 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 substantial 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), both of 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 (e.g., Akt) and their regulators (e.g., PP1 phosphatase). 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 μg) were separated by SDS-PAGE and immunoblotted with various antibodies as indicated. Histone H3 and actin were used as internal standards. Amil, amiloride.
protein kinases (MAPK) by Western blot analysis. The results showed that amiloride downregulated the antiapoptotic proteins Bcl-2 and Bcl-xL in a dose-dependent manner and upregulated the proapoptotic proteins Bax and Bcl-xS (Supplementary Fig. S4A). As expected, the protein expression levels of Bcl-xL and Bcl-xS 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 proapoptotic, with a 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 (Supplementary 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 wanted 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 2 agents produced synergistic effects (Fig. 6A and B). Notably, the addition of 0.05 mmol/L 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 substantial increase in the inhibition of cellular proliferation (Fig. 6C). In addition, the dose–response curve revealed that amiloride concentrations as low as 0.01 mmol/L could increase the growth inhibition effect of 16 μmol/L 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 both inhibit the leukemic cell growth and synergize with imatinib to Bcr-Abl Cells. Moreover, using mononuclear cells from 3 blast-crisis CML patients and 4 healthy individuals, we found that mononuclear cells’ antiviability response to amiloride is substantially lower in CML patients than in health controls (Supplementary 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 the exon exclusion of Bcl-x and HIPK3 with a 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 the dephosphorylation of SR proteins, which in turn played a role in the 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 a PP1-mediated splicing mechanism (27). S-Adenosylmethionine as well as its metabolites is proapoptotic by stimulating the PP1-catalyzed dephosphorylation of RS proteins, resulting in the formation of the alternative variant Bcl-xS (28). Emetine,

![Figure 6. Treatment with amiloride enhances sensitization of CML cells to imatinib. K562 cells (A, B) and BaF3/Bcr-Abl/T315I 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.](image-url)
an inhibitor of protein synthesis, can also regulate the alternative splicing of \( Bcl-x \) transcript to produce proapoptotic \( Bcl-x_S \) 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, particularly cancer, and failure to activate this pathway can result in the resistance of cancer cells to the cytotoxic effects of multiple chemotherapeutic agents. Therefore, shifting splicing toward the proapoptotic isoform offers therapeutic potential in cancer treatment. The splicing of 3 more apoptosis-related genes, \( \text{APAF-1} \), \( \text{CRK} \), and \( \text{SURVIVIN} \), is found to be altered by amiloride. Apaf-1 functions as an adaptor protein during the 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). \( \text{CRK} \) oncogene is spliced into 2 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 the 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 the 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 the modulation of the 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 toward the need to develop novel therapeutic strategies for CML patients. Combining imatinib with other anticancer 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 resensitize BaF3/\( Bcr-AblT315I \) cells to imatinib. These results provide important \( \text{in vitro} \) 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 a targeted therapy drug such as imatinib. The calculated amiloride concentration in the renal distal tubule is approximately 3 to 20 \( \mu \)mol/L (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 mmol/L (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 the 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.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

W-H. Chang designed and performed experiments, analyzed data, and wrote the draft; C-C. Lee, Y-H. Lin, and T-Y. Chen participated in the coordination of the study; T-C. Liu, W-K. Yang, and J-G. Chang conceived/guided the project, and revised the draft.

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