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ARAŞTIRMA / RESEARCH

Effect of sodium butyrate on some alternative splicing genes and BACE1 isoforms in Sh-Sy5y cell line

Sh-Sy5y hücre hattında sodyum bütiratın bazı alternatif kırpılma genleri ve BACE1 izoformları üzeindeki etkisi

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Abstract

Purpose: The aim of this study is to investigate effect of sodium butyrate (NaB), which is a Histon Deacetylase (HDAC) inhibitor, on BACE1 isoforms BACE1/501, BACE1/457 and BACE1/432 and alternative splicing factors including hnRNP H, U2AF35, U2AF65, SRSF1, SRSF2, SRSF5 and SRSF6.
Materials and Methods: In this study, SH-SY5Y cells were treated with of 1 mM and 5 mM NaB. Then Real-Time PCR method was performed to evaluate the change in expression of alternative splicing genes and BACE1 isoforms.
Results: At 5 mM NaB concentration, a relative decrease of 35% to 80% in expression of the SRSF6, SRSF1, SRSF2, SRSF5 and U2AF65 genes were detected. Morever 5 mM NaB concentration increased expression of BACE1/501, BACE1457 and BACE1/432 isoforms. As NaB concentration increase, expression of SRSF6 mRNA decreased to a lower extent than others.
Conclusion: According to our results, it can be concluded that contribution of the U2AF65 and SRSF6 genes to the increase of BACE1/457 and BACE1/432 expression is higher than the other genes.

Keywords:. Alzheimer's disease, alternative splicing, sodium butyrate

Öz

Amaç: Bu çalışmanın amacı, bir Histon Deasetilaz (HDAC) inhibitörü olan Sodyum Bütirat'ın(NaB) BACE1/501, BACE1/457 ve BACE1/432 gibi BACE1 izoformları ve hnRNP H, U2AF35, U2AF65, SRSF1, SRSF2, SRSF5 ve SRSF6 alternatif kırpılma faktörleri üzerindeki etkisini araştırmaktır.
Gereç ve Yöntem: Bu çalışmada, SH-SY5Y hücreleri 1 mM ve 5 mM Na Bile muamele edilmiştir. Daha sonra BACE1 izoformları ve alternative kırpılma genlerinin ekspresyonlarındaki değişimleri değerlendirmek için Real-Time PCR yöntemi kullanılmıştır.
Bulgular: 5 mM NaB konsantrasyonunda SRSF6, SRSF1, SRSF2, SRSF5 ve U2AF65 genlerinin ekspresyonlarında %35'ten %80'e kadar görece bir azalma olduğu belirlenmiştir. Ayrıca 5 Mm NaB BACE1/501, BACE1/457 ve BACE1/432 izoformlarının ekspresyonunu artırmıştır. NaB konsantrasyonu arttıkça, SRSF5 geninin ekspresyonu diğerlerinden daha fazla azalmıştır.
Sonuç: Bulgularımızdan yola çıkılarak, U2AF65 ve SRSF6 genlerinin BACE1/457 ve BACE1/432 izoformlarının artışına diğer genlerden daha fazla katkı sağladığı sonucuna varılmıştır.

Anahtar kelimeler: Alzheimer hastalığı, alternatif kırpılma, sodyum bütirat

INTRODUCTION

Alzheimer Disease (AD), which is the most common form of dementia, is caused by many risk factors such as aging and decline of cognitive function in central nervous system[1]. AD, which affects nearly 35.6 million people worldwide, has been characterized as a complex disease with interaction of genetic background, environment and the life style[2]. The

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most typical hallmark of AD is the formation of amyloid β protein and accumulation of it as a plaque in the brain. Amyloid β is formed by proteolytic clevages of several proteolytic enzymes from a precursor protein called as Amyloid Precursor Protein (APP). APP can be cleaved by two different pathways. While one of them is Amyloidogenic pathway, the other is Non-amyloidogenic pathway. After APP is cleaved by β-secretase in amyloidogenic pathway, resulting proteins are APPs β (soluble APP β) and C99, a membrane-bound carboxyl terminal fragment. C99 is then cleaved by γ-secretase and therefore Amyloid β fragment is formed and released from membrane. After Amyloid β is formed, it deposits in the brain. After APP is cleaved by α-secretase in non-amyloidogenic pathway, resulting proteins are APPs α (soluble APP α), and C83. Then C83 is cleaved by γ-secretase and P3 and APP intracellular domain (AICD) are formed[3].

Premature mRNAs processing with alternative splicing, increases coding capacity of the human genome. Alternative splicing occurs with the help of a machinery called splicesome which is composed of 5 small nuclear RNA (snRNA) molecules known as U1, U2, U4, U5, U6 and a variety of protein molecules. U2 Small Nuclear Ribonucleoprotein Auxillary Factor (U2AF) heterodimer consists of two subunit called as U2AF35 (35 kD small subunit) and U2AF65 (65 kD large subunit) and binds to the polyprimidine tract[4].

Alternative splicing can be regulated via cis-acting sequence elements or trans-acting factors. While cis-acting sequence elements are in pre-mRNA sequence, trans-acting factors which are proteins known as Serine-Rich proteins (SR) and heterogeneous nuclear ribonucleoproteins (hnRNP) recognize spesific sequences in pre-mRNA sequence and bind to it[5]. Alternative splicing pattern could be changed by depending on tissue type, development stage or disease situation. Alternative splicing is commonly observed in the brain. Therefore, it can be explained that alternative splicing contributes to complexity of the brain. Expression changes occured both hnRNP and SR proteins can lead to disturption in alternative splicing of many genes[4].

BACE1 enzyme also known as a β-secretase has nine exons and undergoes alternative splicing[6]. Full-length 501-aa protein (BACE1/501) is produced by normal splicing in which introns are removed and exons are joined to each other. Alternative splicing of BACE1 enzyme takes place in alternative splicing regions within exon 3 and exon 4. Three isoforms are produced through different combination between alternative splicing sites and normal splicing sites. These isoforms are called as BACE1/476, BACE1/457 and BACE1/432 splice variants according to amino acid numbers. These isoforms were found firstly in pancreas. BACE1/501 has a full catalytic activity. However, splice variants lack full catalytic activity. It has been shown that splice variants do not lead to deposit of amyloid β. It is thought that an inducible increase in the expression of variants can be therapeutic. Therefore, effect of alternative splicing on BACE1 enzyme involved in AD pathogenesis is important[7].

Many splicing factors can bind to pre-mRNA and can affect alternative splicing of it[8]. A G-quadruplex structure in BACE1 pre-mRNA plays a role in selection of the splicing site[9]. HnRNP H, belong to a ribonucleoprotein involved in splicing process, binds to Guanine-rich sequence, which form a G-quadruplex structure. BACE1 has also G-quadruplex in exon 3 and hnRNP binds to the motif to form 501-length isoform. Inhibition of hnRNP H leads to a decrease in expression of BACE1/501, full length form of enzyme, which is responsible for AD and therefore leading to accumulation of amyloid β protein[10]. Pre-mRNA of BACE1 also has a binding site for SRSF2 and inclusion of the exon 4 is regulated by the binding site[11].

It also has been determined that while hnRNP H prefers to bind GGGGG motif, SRSF1 prefers to bind GGAGG motif. It has been found that these two splicing factors have an antagonistic effect to each other[12]. In addition, it has been demonstrated that U2AF65, a splicing factor involved in alternative splicing, interact with the G-quadruplex structure in BACE1 pre-mRNA[11].

Histone deacetylation, occurred by Histone deacetylase enzymes (HDACs), generally causes the gene silencing. These enzymes remove acetyl group in lysine residue on histone proteins associated with DNA molecule. Because DNA is packed more tightly, transription factors do not reach genes. Therefore, the genes are not expressed[13]. Histone deacetylase inhibitors can lead to expression of some target genes. Recently, HDAC inhibitors such as valproic acid, trichostatin A, sodium-phenil butyrate and vorinostat have been developed with therapotic aims for some complex diseases. Histone deacetylase inhibitors has been used in treatment of cancer and neurodegenerative diseases[14].

In a study, it has been shown that HDAC inhibitors improve cognitive function in AD mouse models. Therefore, it has been thought that HDAC inhibitors can be alternative drugs for patients with AD. Moreover, valproic acid decreased memory problems and Amyloid β plaque by inhibiting γ-secretase enzyme in transgenic mouse models of AD. However, it is necessary to enlighten HDAC-associated cellular signalling mechanisms in AD pathogenesis. Sodium butyrate (NaB) is a short chain fatty acid and dietary histone deacetylase inhibitor[15]. It is reported that NaB causes induction of apoptosis and inhibition of cell growth in some cancers such as breast, colon and cervical cancer cell lines[16,17]. In another study, it has been determined that when transgenic mice which show aggressive pathogenesis of AD treated with NaB, it reversed memory problems of the mice and increased the expression of genes related to learning. Moreover, it has been found that NaB decreased the expression of splicing variant of tau which responsible for neurofibrillary tangles in AD pathogenesis. Also HDAC inhibitors can affect alternative splicing of many genes[18].

Although SH-SY5Y cells are bone-marrow derived cells taken from a petient with neuroblastoma, SH-SY5Y cells have been utilized for in vitro experiments requiring neuronal-like cells[19]. Therefore SH- SY5Y cell line is usefull as a cellular model for biochemical investigations on AD[20].

We aimed to investigated the effect of NaB on BACE1 isoforms such as BACE1/501, BACE1/457 and BACE1/432 and alternative splicing factors such as hnRNP H, U2AF35, U2AF65, SRSF1, SRSF2, SRSF5 and SRSF6 in our study. We examined expressions of BACE1/501, which is responsible for AD and the other isoforms which do not cause accumulation of amyloid β. Also we investigated expression changes in splicing factor genes especially involved in BACE1 splicing after treatment of NaB, a histone deacetylase inhibitor.

MATERIALS AND METHODS

Cell culture

The study was approved by Çukurova University Faculty of Medicine Non-Invasive Clinical Research Ethics Committee (02.10.2015/46). SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 1:1 added %10 Fetal Bovine Serum (FBS), %1 Penicillin-streptomycin and %1 L-glutamine. Cells were cultivated at 37°C with %5 CO_2. Cells were seeded at an initial density of 250.000 cells cultured in 6-well plate per well. Then, after cell confluency reached %75 level in each well, cells were treated with NaB.

Sodium butyrate treatment

Sodium butyrate (Across organics) was dissolved in DMSO (final DMSO rate in cell culture dishes = %0.02) Final concentration of NaB added to culture dishes was 1 mM and 5 mM. Cells were treated with NaB for 24 hours. These doses were chosen according to results of some studies[21]. In this studies, when cancer cells were treated with 1 mM and 5mM doses for 24 hours, rates of apopotosis were low. Because neuronal apoptosis plays an important role in AD pathogenesis, in our study low rate of apopotosis is very important for us[22].

Control treatment

For control group, cells were treated with DMSO for 24 hours. Final DMSO rate in cell culture dishes was %0.02, because neurotoxic level of DMSO in SH SY5Y is about %0.02.

RNA isolation

Total RNA from control group cells and experiment group cells treated two doses of NaB was extracted by Trizol Reagent (Sigma) according to the manifacturer's instructions. Purity and concentration of total RNA were measured by Nanophotometer (IMPLEN). Samples which A260/A280 and A260/A230 ratios are ~2.0 were used for following procedures.

cDNA synthesis

cDNA Synthesis Reactions were done by High-Capacity cDNA Reverse Transkription Kit (Applied Biosystem) according to the manifacturer's instructions. 1,5 µg RNA sample was used 20 µl per reaction.

Real time PCR reaction

Real-Time reactions were carried out by Power Sybr Green master mix (Applied Biosystem). Reactions were performed by Applied Biosystem 7500 Real-Time PCR system. Primers used in Real-Time PCR reactions were designed by Primer-BLAST. GAPDH was used as a reference gene. The primers are listed in Table 1. PCR cycling condition as follow: for

holding stage (1 cycle); 2 min. 50 °C, 10 min. 95 °C. For cycling stage (40 cycle); 15 sec. 95 °C, 1 min. 60 °C. For melting curve stage (1 cycle); 15 sec. 95°C, 1 min. 60°C, 30 sec. 95 °C, 15 sec. 60 °C.

Table 1. Used primer sequence in RT-PCR reactions

GAPDH	F: AGGGCTGCTTTTAACTCTGGT
	R: CCCCACTTGATTTTGGAGGGA
HNRNP H	F: GTAACCAAGGAGCAGTGAACAG
	R: AGGTTGACCAAGAGTCAGTG
U2AF35	F: AGTAGAGGAGATGAACGTCTGTG
	R: GGATCGGCTGTCCATTAAACC
U2AF65	F: AATAAACAAGAGCGGGACAAGGA
	R: AAAGGTTTGCTGCGTCGTC
SRSF1	F: ACCTCCAGACATCCGAACCA
	R: CGAACTCAACGAAGGCGAAG
SRSF2	F: GTGGACAACCTGACCTACC
	R: AAAGCGAACGAAGGCGAAG
SRSF5	F: AGGGAGAAGGACGTGGAAAGA
	R: CGAGCCCTAGCATGTTCAATAG
SRSF6	F: TGGACAAACTGGATGGCACA
	R: CCTGCGACTCCTACTTCGTG
BACE1/501	F: TCTTCATCAACGGCTCCAACT
	R: GAGAAGAGGTTGGGAACGTG
BACE1/457	F: GGAAGGGTGTGTATGTGCCC
	R: GAGTCGTCAGGCAGGTCG
BACE1/432	F: GGAAGGGTGTGTATGTGCCC
	R: CACCACAAAGCAGGTCGGT

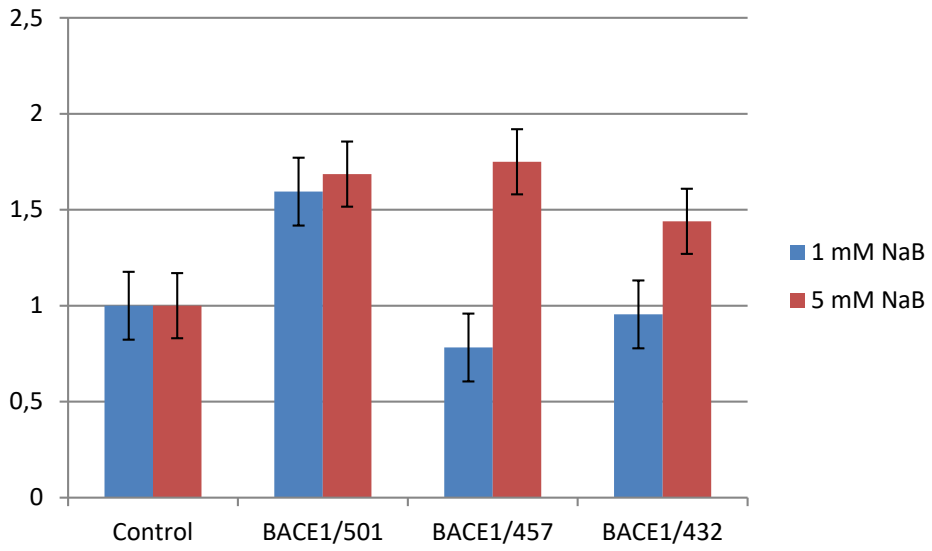

Figure 1. Expression changes of BACE1 isoforms after NaB treatment.

Statistical analysis

The threshold cycle (Ct) numbers were determined and expression changes were calculated through ΔΔCt method. All experiment sets were repeated three times. Avarage of expressions were calculated and statistical analyses were carried out by SPSS 22 version (IBM). Statistical test used to compare significance between doses of NaB is Mann-Whitney U test. Statistically significant differences were determined by p values less than 0.05 ($p<0.05$).

RESULTS

After SH-SY5Y cells were treated with 1 mM or 5 mM NaB, Real-Time PCR was performed to determine the mRNA expression changes of alternative splicing related genes such as, hnRNP H, U2AF35, U2AF65, SRSF1, SRSF2, SRSF5 and SRSF6 along with BACE1 isoforms including BACE1/501, BACE1/457 and BACE1/432. Statistically significant difference between two doses of NaB has not been found ($p>0,05$). 1mM and 5mM NaB increased BACE1/501 expression compaired to control group. While 1mM NaB decreased BACE1/457 expression, 5 mM NaB increased BACE1/457 expression. 5 mM NaB increased BACE1/432 expression compared to control group (Figure 1).

Although it was not statistically significant in hnRNP H, U2AF35, U2AF65, SRSF1, SRSF2, SRSF5 and SRSF6 expressions to Mann-Whitney U test, it was observed relative increase in expression of U2AF35 and SRSF6 genes and relative decrease in hnRNP H, SRSF1, SRSF2, SRFSF5 and U2AF65 mRNA expressions after 1 mM NaB treatment. In addition, it was identified a decrease of %40-50 in the genes expression in 1 mM NaB (Figure 2). In 5 mM NaB concentration, it was determined relative decrease from %35 to %80 in expression of SRSF6, SRSF1, SRSF2, SRSF5 and U2AF65 genes, respectively (Figure 2).

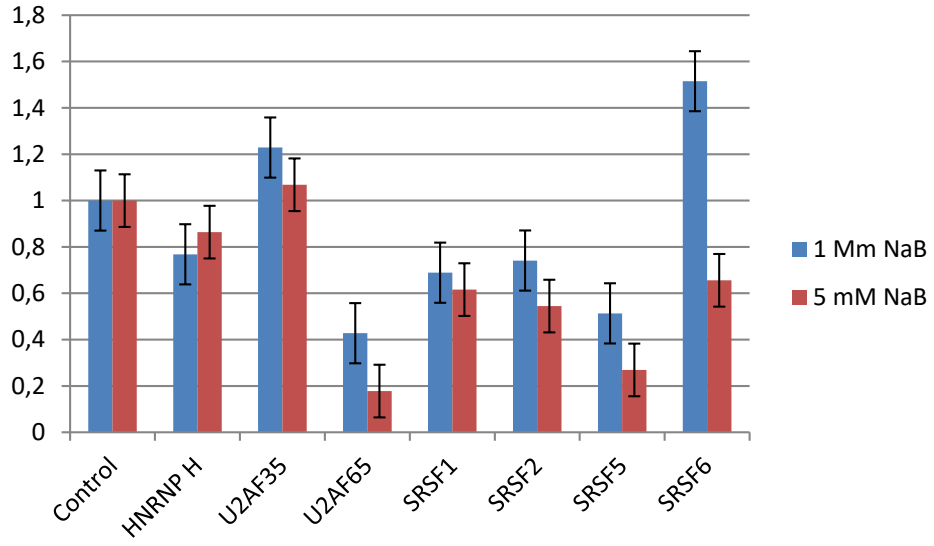

Figure 2. Expression changes of the alternative splicing-related genes after NaB treatment

DISCUSSION

Chromatin modifications have important roles in pre-mRNA splicing. It has been reported that alternative splicing of several genes is regulated by HDAC activity. In the HeLa cell line, the acetylation of SRSF5, a splicing regulator, has been shown to be altered by HDAC inhibition[18]. In addition, a recent study has demonstrated that treatment of NaB

increases expression of a splicing variant of glucocorticoid receptor gene in lymphoma cell line[23].

Recent studies determined that NaB administration reduces the effect of amyloid β which is responsible for an important part of Alzheimer's disease and leads to cognitive improvement in transgenic mice[24]. In our study, NaB increased expression of BACE1 / 501 as opposed to anticipated. Given that the SH-SY5Y cells used in this study are neuron-like cells, the increase in expression of BACE1/501 may be thought to be due to the dominant effect in the strong 5' regulatory region, which plays an important role in neuronal cell processes such as myelination[7].

Our main goal was to investigate effect of NaB as a chemical molecule on BACE1 isoforms by determining changes in expression profile of alternative splicing. 5 mM NaB has caused a noticeable change in expression of BACE1/457 and BACE1/432 isoforms (Figure 2).

BACE1/501 expression was increased in both doses of NaB (1 mM and 5 mM), but isoforms such as BACE1/457 and BACE1/432, especially in 5mM NaB, also increased notably as an indicator of increased alternative splicing. In our questions about which splicing factors may be responsible for the increase of these isoforms, we have reached the results we discussed below.

Recent studies demonstrated that hnRNP H increases the expression of BACE1/501 by binding to G-quadruplex in the exon 3 of BACE1[11]. Thus, the increase in expression of BACE1/501 in our study may be due to the non-reduction of hNRNP H expression. Moreover, in our study we observed that the expression of hNRNP H did not change in either doses of NaB. Therefore, we believe that the increase in expression of BACE1/457 and BACE1/432 as NaB doses increase (especially 5mM NaB) is caused by the other splicing factors other than hnRNP H.

There are different studies about effect of U2AF65. In a study targeting genes including SMN and tau have shown that inhibition of U2AF65 gene causes directional exon skipping[25]. However, in our study it can not be thought that U2AF65 gene is responsible for increase in BACE1/457 and BACE1/432 isoforms by exon skipping mechanism because production of these isoforms is associated with usage of 3' and 5' splicing sites rather than exon skipping[8]. In other studies, it has been reported that U2AF65 binds to G-rich sequence and the deletion of the G-rich regions (G-quadruplex) in the exon 3 of the BACE1 gene increases the formation of BACE1/457 and BACE1/432 while reducing the formation of BACE1/501[11]. In our study, as the NaB concentration increased, the expression of BACE1/457 and BACE1 / 432 increased, the expression of hNRNP H and BACE1 genes remained unchanged, and the expression of U2AF65 gene decreased. We have considered that decrease in expression of U2AF65 gene causes increase in expression of BACE1/457 and BACE1/432 isoforms by inducing alternative splicing of BACE1 pre-mRNA. Studies to determine the role of U2AF65 in the identification and binding of the splicing site have also shown that, several genetic diseases, such as Retinis pigmentosa and Neurofibromatosis, are associated with splicing binding site mutations of U2AF65 and the production of alternative protein isoforms. In addition, U2AF65 has been found to have a decreased affinity for its target in the case of a mutation in the polypyrimidine tracts in the target sequence of U2AF65[26]. As a matter of fact, we have concluded that inhibition of NaB on U2AF65 promotes production of isoforms through different alternative splicing mechanism such as exon exclusion in 3' and 5' alternative splicing sites because our study has not included mutation of the genes involved in alternative splicing.

SR proteins have regulatory function by binding to RNA motifs in both constitutive and alternative splicing[5]. In some diseases such as Myelodysplastic syndrome, changes in the binding affinity to motif sequences in the target transcript have been identified due to mutations such as P95H in the gene encoding SRSF2[27]. It has also been shown that exonic splicing enhancer sequences in the exon 4 of BACE1 pre-mRNA are also recognized by SRSF2 and that SRSF2 regulates alternative splicing products[28]. However, due to the relative decrease in SRSF2 due to increased NaB concentration in our study, we have concluded that SRSF2 may not be responsible for the increase in alternative splicing isoforms. SRSF2 and other SR proteins have 2 domains; while one of them is sequence-specific RNA binding domain in N-terminal, the other regulates interaction with the other splicing factors in C-terminal[29]. For these reasons, we can not say for sure whether the increase in BACE1 isoform we detected in our study is due to the interaction of the C-terminal domain of SRSF2 with other splicing factors or not.

A study showed that SRSF1 and hnRNP H have antogonistic effect in alternative splicing and they

bind to similar motifs[13]. In our study, increase in NaB concentration did not induce any changes in the expression of SRSF1 and hNRNP H in the SH-SY5Y cell line. Therefore, we speculated that the NaB has not affected the balance between SRSF1 and hnRNP H.

SRSF6, another factor we have associated with the increase in BACE1/457 and BACE1/432 in our study, has been shown to have antagonistic effect on other SR proteins by negatively regulating the 5 ' splicing site[30]. In our study, we found decrease in expression of SRSF1, SRSF2, SRSF5 and SRSF6 genes. Since the expression of SRSF6 is the most reduced among these genes, the increase in the production of BACE1 isoforms is thought to be the dominant effect of SRSF6 relative to the others. We considered that decrease in expression of SRSF6 gene based on NaB dose promotes alternative splicing of 5' splicing site of the exon 3 on BACE1 pre-mRNA and therefore leads to increase in expression of BACE1 isoforms. This conclusion needs to be supported by future studies. If such a dominant SRSF6 effect is detected for the BACE1 gene, a therapeutic approach to Alzheimer's disease targeting SRSF6 inhibition may be successful instead of non-specific histone deacetylase inhibitors such as NaB, valproic acid, etc. Currently, drugs targeting histone acetylation / deacetylation make an effort to use in the treatment of some complex diseases such as cancer. However, one of the disadvantages of this strategy is that it occurs genome-wide instead of targeting specific genes[31]. It is not clear which genes are expressed by this hyperacetylation caused by HDAC inhibitors but it is speculated that this may have an effect on nearly 2% of the genome[32]. A limitation of this study is about inhibition of histone deacetylation which has low specifity in genome-wide. We considered that we need to focus on chemicals specifically targeting of alternative splicing genes on BACE1 pre-mRNA in spite of inhibition of histone deacetylation in genome-wide.

Yazar Katkıları: Çalışma konsepti/Tasarımı: GA, ÜL; Veri toplama: GA, GE; Veri analizi ve yorumlama:GA, ÜL; Yazı taslağı: GA, PP; İçeriğin eleştirel incelenmesi: ÜL, PP, GA; Son onay ve sorumluluk: ÜL, GA, GE, PP, DA, AP, MBY; Teknik ve malzeme desteği: AP; Süpervizyon: ÜL; Fon sağlama (mevcut ise): yok.
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