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NF-κB/RELA Knockout Reveals a Role in Expression of HIF-1α and NES in A172 Cell Line: Suggestion Ecteinascidin-743 as a Suitable Drug

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ABSTRACT

The NF-κB signaling pathway is one of the most critical controllers of the inflammatory response in glioblastoma multiforme (GBM). In this study, the knockout association of the RELA subunit was examined by expressing some genes connected to the nuclear factor κB (NF-κB) pathway in sustaining hypoxic circumstances and GBM stem cells (GSCs) properties. A suitable drug is also suggested to target RELA **.** Targeted deletion of the RELAsubunit in human GBM cells (A172) was performed with CRISP R/Cas9. Nestin (NES) and hypoxia inducible factor 1 alpha (HIF-1α) genes expression levels and induction of apoptosis in transfected and control cells were assessed by Real-time PCR and flow cytometry, respectively. Using the Auto Dock Vina software, a molecular docking study was conducted to find the optimum intermolecular interaction between the RELA protein and four potential drugs. Real-time PCR results showed a decrease in the expression of HIF-1α and NES genes in the transfected cell population compared to the control cells ($p< 0.0102$, $p< 0.0012$, and $P< 0.0442$, respectively).

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Flow cytometry results showed a significantly increased induction of apoptosis in the transfected cells compared to the control cell population. The results of docking revealed that Ecteinascidin-743 has the best intermolecular interaction with RELA protein. In conclusion, the RELA subunit seems to be one of the factors affecting hypoxia, apoptosis, and change in stemness gene expression levels in GBM**.** Therefore, it is recommended to knock out the NF-κB signalling pathway or use Ecteinascidin-743 in future studies.

Keywords: *Glioblastoma multiforme; NF-κB; RELA*; CRISPR/Cas9; Ecteinascidin-743

1. INTRODUCTION

Glioblastoma multiforme (GBM) is an invasive anaplastic astrocytoma, the most malignant form of glioma with a 15-month median survival of patients after diagnosis. The high degree of heterogeneity, therapeutic resistance, and a high recurrence rate ofGBM are attributed to the presence of GSCs (Gimple et al., 2019; Pesenti et al., 2019). The cell population observed in the hypoxic niche that is highly dependent on the ,tumour microenvironment to maintain its stemness self-renewal, multipotentnature, and treatment resistance (Filatova et al., 2013). GBM is determined as a hypoxic inflammatory brain tumour with a vascular system and necrosis-extensive areas and high angiogenesis. Inside the tumour microenvironment on the one hand, stabilizing HIF-1α as the key hypoxia regulating factor leads to tumour cell adaptation to hypoxia and enhanced angiogenesis. On the other hand, it induces necrosis and the release of alarmins, which then directs the expression of proinflammatory cytokines and chemokinesin response to NF-κB activation. Eventually, it results in the occurrence of chronic inflammation and the utilization of cell types and signalling pathways (Papale et al., 2020; Tafani et al., 2011). NF-κB is a major regulator of the immune response and inflammation and is a major cancer-related factor. This transcription factor plays an important role in a variety of clinical and physiological processes, including the establishment and maintenance of an inflammatory tumour microenvironment.

It is also a crucial regulator of pro-inflammatory cytokine release by tumour and non-tumour cells, particularly tumour-associated macrophages (TAMs). besides, It has a critical role in triggering tumorigenesis in the cellular response to changes in the tumour microenvironment caused by inflammatory mediators (Hoesel and Schmid, 2013; Kaltschmidt et al., 2019). This transcription factor's activity has been found to be elevated in a variety of malignancies under various physiological situations (Gilmore, 2021). In GBM, the NF-κB signalling pathway isaberrantly active and the RELA subunit is often phosphorylated. Numerous studies have also shown NF-κB activation in patient-derived glioblastoma stem cell cultures medium (Friedmann-Morvinski et al., 2016; Soubannier and Stifani, 2017). The role of the NF-κB signalling pathway in the pathogenesis of GBM is well understood and considered a potential target for the treatment of GBM. Designing treatment strategies by targeting tumour microenvironment components or regulatory signalling pathways to modulate the hypoxic and inflammatory responses is considered a beneficial strategy. With regards to enhancing the tumorigenic potential of cancerous cells through crosstalk between signalling pathways including hypoxia, inflammation, and stemness genes, the present study was focused on the specific knockout of the RELA subunit using the CRISPR/Cas9 system. After that, we evaluated the expression levels of HIF-1α and NES genes and the rate of induced apoptosis in the A172 cell line compared to non-transfected cells. Moreover, due to the importance of this signalling pathway in GBM stemness properties, appropriate drugs have been suggested for further studies

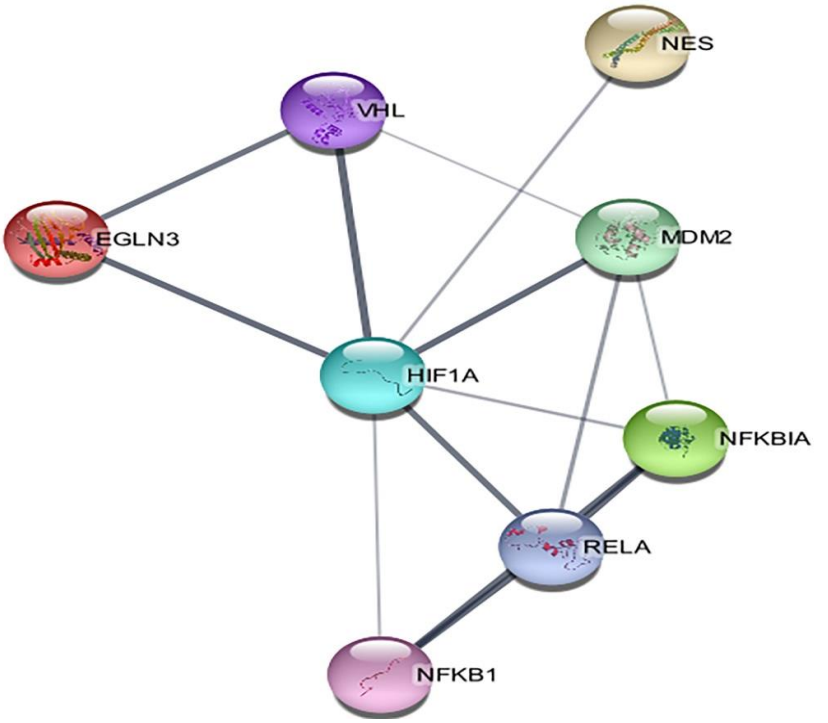

FIGURE 1. Crosstalk between selected genes in this study: RELA **HIF-1α** and NES genes. Cytoscape_v3.8.2.

MATERIALS AND METHODS

CRISPR/Cas9-mediated RELA gene knockout

The strategy used for knockout of the RELA gene involves induction of two double-strand breaks in two different coding sequences followed by the deletion created in the gene. For this purpose, the two CRISPR/Cas9 plasmids (pSpCas9 (BB)-2A-Puro (PX459) V2.0) were used in our previous study. Both utilized plasmids for genome editing were separately amplified in the Stbl4 strain of E. coli bacteria according to the cacl2 method, followed by the plasmids being extracted and purified from the bacteria using the plasmid DNA purification kit. The quantity and quality of the extracted plasmids were evaluated by Thermo Scientific ™ NanoDrop and electrophoresis on 1% agarose gel.

Cell culture and transfection of PX459 CRISPR/Cas9 cut sites

A172 (CRL-1620™) as an adherent cell line was purchased from the National Cell Bank of Iran (NCBI). This cell line was cultured under inappropriate cell culture media, RPMI 1640 medium (# 31800-022, Gibco) supplemented with FBS (# 10270 %10-106, Gibco) and 1% penicillin-Streptomycin (# 15140122, Gibco), and was kept at C temperature in a humidified CO2° 37 .incubator A172 cells were co-transfected with both PX459 CRISPR/cas9 vectors at the same time using mediated) Lipofectamine TM 3000Transfection Reagent Green Catalog number: ,(L3000001 according to the kit manufacturer's protocol and under FBS-free DMEM media. conditions

The next day, the cellular medium was replaced with a fresh DMEM containing 20% FBS. After 24 hours to select transfected positive cells, puromycin with a final concentration of 2 mg/ml was added for days. Followed by, the growth conditions of the 3 remaining cells, for carrying out further studies, were provided.

To confirm transfection of A172 cells with PX459 CRISPR/cas9 and evaluate the function of plasmids in causing a 180 bp deletion in the RELA geneamplifying the regions containing the cut sites by using specially designed primers was .carried out In order to, genomic DNA of transfected and untransfected A172 cells was isolated using a DNA extraction kit (DNA Extraction Kit, Sinaclon Cat No. EX6071) for PCR reaction

RNA isolation and Real-time PCR

According to existing standard protocols total RNA was isolated from transfected and control A172 cells using phenol-chloroform-based RNXTM (plus) Solution. cDNA synthesis of each sample with a concentration of 1000ng RNA was done in accordance withthe protocol of the cDNA synthesis kit(Yekta Tajhiz Azma, Iran #YT4500 .) A quantitative real-time PCR was performed by Rotor-Gene 6000 (Corbett Research, Sydney, AU), using specific primers for each gene, and a 2X Super SYBR Green qPCR Master Mix kit (Yekta Tajhiz Azma, Iran #YT2552) was used according to the protocol provided by the manufacturer. All primer sequences that were used for the RT-PCR assay, are listed in Table 1. The relative levels of mRNAs expression were normalized by GAPDH expression as an internal control (2-ΔΔCt).

Apoptosis assay

The rate of induction of apoptosis was investigated in the transfected and untransfected cells.

For apoptosis analysis, 1 x 106 cells were labeled with Annexin V-FITC/PI (BD Biosciences cat: 51-66211E) according to the manufacturer's instructions, followed by the percentage of apoptotic cells were measured using flow cytometry (Partec sysmex, Germany) in transfected and control cells. FlowJo.V10 software (Tree Star, Ashland, OR, USA) was used to analyze the data.

Protein and ligand retrieval

The amino acid sequence of RELA protein was retrieved from UniProt. The 3D structure of the protein was predicted by using the Swiss model server. In addition, the molecular structures of drugs were retrieved from the PubChem data source in the .sdf format (Kim et al., 2016)

Molecular docking

The binding mode of the target protein with drug candidates was identified through the molecular docking study by using the Auto Dock Vina virtual screening software (Trott and Olson, 2010). Auto Dock Vina is a molecular docking tool having an easy-to-use user interface. The best binding poses of the protein and ligand were identified based on the highest binding energy (kcal/mol) with the negative sign. The generated protein–ligands complex through the docking study was further investigated by visualizing its structure using the BIOVIA Discovery Studio Visualizer v19.1.0.18287 (BIOVIA) (Ahammad et al., 2019).

Statistical analysis

All statistical analyses of Real-time PCR and flow cytometry results were evaluated using GraphPad Prism software version 8.4.3 GraphPad) Software, CA). According to the normal distribution of data, an independent t-test was used for data with normal distribution to compare the quantitative variables between the two transfected and control groups

The diagrams of quantitative data were presented by GraphPad Prism software version 8.4.3 GraphPad) Software, CA). Variables with a normal ."distribution were provided as "mean ± SEM P<0.05 was consideredas a significant difference.

RESULTS

According to corroborate the quantity and quality of extracted CRISPR/Cas9 plasmids, transfection on A172 cells was accomplished Followed by adding puromycin in cell culture media and observing the death of untransfected cells over days, the growth 3conditions of the remaining cells were provided. Subsequently, confirming of transfection A172 cells with PX459 CRISPR/Cas9 was accomplished (Supplementary file 1).

Real-time PCR results

Sought to explore the potential effects of RELAknockout to block ing the NF-κB pathway as a therapeutic approach on A172 cells, we examined the expression level of the HIF-1α gene, as the main regulator of tumor hypoxia and angiogenesis, and NES as a stem cell marker, on the transfected and control cellsAs s hown in figure2, our result of the Real-time PCR analysis reveals the expression levels of the HIF-1α and NES genes were significantly decreased by 1.65- and 2.18 folds in the transfected cells compared to the ,control group .respectively

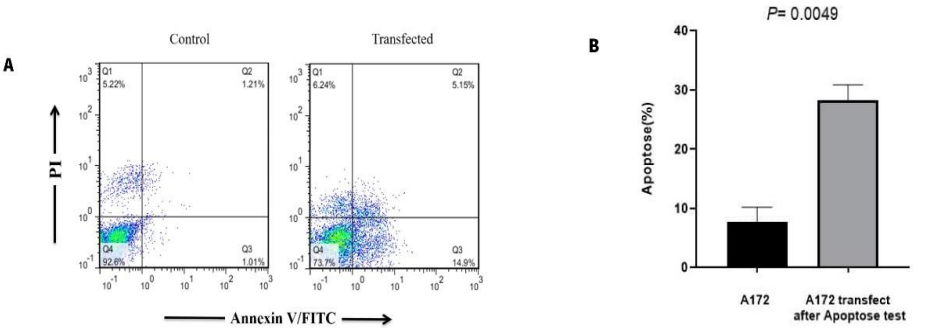

FIGURE 2. The results of CRISPR/Cas9-mediated knockout of RELAon HIF-1α and NES genes expression. A) The results show decreased expression of the HIF-1α gene (2.5-fold) in the transfected cells compared to the control cell population. (p = 0.0012) B) The result show decreased expression of the NES gene (1.25-folds) in the transfected cells compared to the control cell population(P= 0.0442) All Data are presented as mean± SD, n = 3 p < 0.05

Apoptosis assay

As shown in Figure 3, there is a significant difference in the rate of induced apoptosis in cells transfected with CRISPR/Cas9. These findings suggest that NF-κB inhibition causes apoptosis

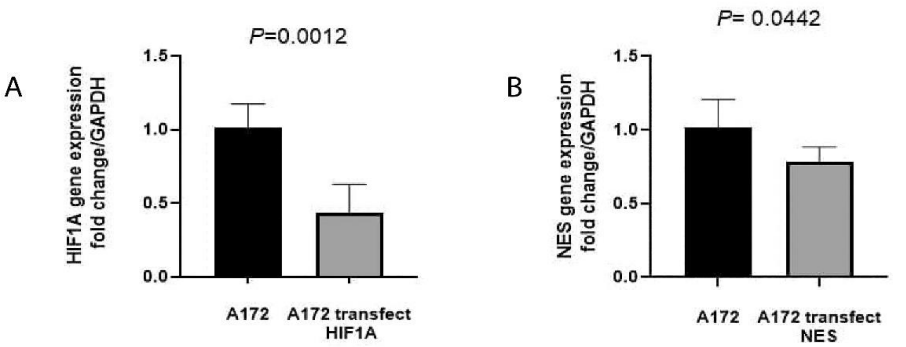

FIGURE 3. A) The fluorescence curve was showed an increase in the percentage of apoptotic induction in CRISPR/Cas9-transfected cells compared to untransfected cells. B) The rate of induced apoptosis showed a significant increase in the transfected group compared to the control group (P< 0.0049). The number of cells undergoing apoptosis expressed as a percentage relative to the total cell number is indicated. Data expressed as mean ± SD, n =3. p value < 0.05.

Protein and ligand retrieval

The amino acid sequence of RELA (UniProtKB - Q2TAM5) was used to model the 3D structure. The 3D structure of the protein was predicted by using the Swiss model server. Furthermore, the structures of Bortezomib (PubChem CID: 387447), Chromomycin A3 PubChem C)ID: 656673), Digitoxin (PubChem CID: 441207) and Ecteinascidin-743 (PubChem CID: 372978) were retrieved from the PubChem data source.

Molecular docking analysis

A molecular docking study is generally used to identify the best intermolecular framework formed between a small molecule as a drug and a macromolecule as a target protein (Masoudi-Sobhanzadeh et al., 2021; Parvizpour et al., 2021). Accordingly, the best intermolecular interaction between the target protein and four drug compounds was determined based on the molecular docking study by using Auto Dock Vina. The results in Table 2 indicates that the binding affinity of Ecteinascidin-743 (-14.3) is better than Bortezomib (-7.3), Chromomycin A3 (-7.8) and Digitoxin (-9.2). Also, the details of these interactions are shown in Figure 4.

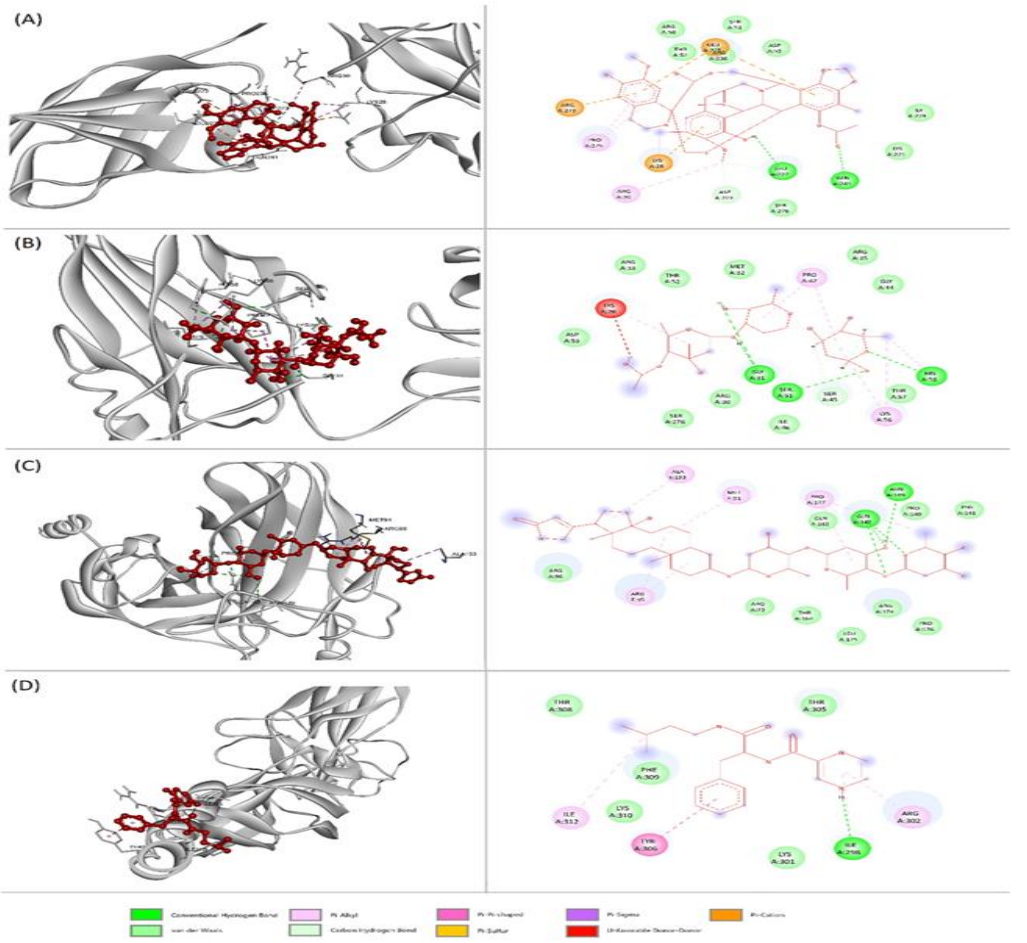

FIGURE 4. 3D (left) and 2D (right) images of the model protein's protein-ligand interaction with Ecteinascidin-743 (Pubchem CID: 372978), Chromomycin A3 (Pubchem CID: 656673), Digitoxin (Pubchem CID: 441207), and Bortezomib (Pubchem CID: 387447).

DISCUSSION

According to studies on cancer patients, hypoxic tumors, such as glioblastoma, are considered a therapeutic problem. Given recent developments, the nature of the tumor microenvironment plays an important role in determining the fate of GSC. Existing inflammation and factors caused by tumor cells can directly alter the tumor microenvironment (Broekman et al., 2018; Sharma and Shiras, 2016).

One of these factors is the NF-κB transcription factor.

Accordingly, this study was performed to inhibit the NF-κB signaling pathway by knockout of the RELA subunit gene. Focusing on the effects of this signaling pathway, the relationship between RELAgene knockout and HIF-1α and NES gene expression levels was investigated. Consistent with previous studies, our results showed a significant reduction in HIF-1αNES genes expression following the knockout of this gene in the transfected group compared to the control group.

Following the suppression of NF-κB action by a range of pharmacological and genetic inhibitors, previous research studied and validated the involvement of this transcription factor and its association with various aspects of carcinogenesis. The results of previous studies confirm the key role of the NF-κB signaling pathway in the process of .induction of angiogenesis in GBM In addition, a connection between NF-B and HIF-1α factors is obvious in tumor-associated inflammation; The hypoxic response is mediated by HIF-1α in an NF-κB-dependent manner (Cahill et al., 2015; Khandia (and Munjal, 2020

In line with the importance of the NF-kB signaling pathway in the growth and survival of GBM cells through the maintenance of GSCs; Rinkenbaugh AL et al. (Rinkenbaugh et al., 2016) following the siRNA-mediated deletion of the RELA gene demonstrated increased phosphorylation of the RELA subunit in +CDM3 ,GBM cells compared to CD133-cells acceleration of differentiation, and disruption of GBM formation and development. GSC CD133, and NES have previously been demonstrated to have promising potential as new forms of prognostic indicators in glioma. NES expression increases significantly as glioma progresses, particularly in GBM (Ishiwata et al., 2011; Jinet al., 2013). In this regard, Nogueira L et al. (Nogueira et al., 2011) observed comparable outcomes in xenografted mice by administering a powerful IKK2 inhibitor and reducing the expression of stem markers like CD133 and NES. As a result, the decrease in expression seen in the HIF-1α and NES genes is consistent with previous research

The rate of induced apoptosis was also measured in this study when the RELA gene was knocked out. Increased apoptosis in the transfected group compared to the controlgroup confirms the involvement of NF-κB in GBM cell growth and survival and is consistent with prior findings. The growth and development of GBM cells can thus be regulated by controlling NF-κB related genetic factors.

TAM, the main component of TME by secreting NF-κB-mediated pro-inflammatory cytokines plays a critical role in the development of inflammatory TME and angiogenesis, as well as the suppression of the anti-tumor immune response (Lawrence, 2010). The results are in line with the study conducted by Achyut BR et al. (Achyut et al., 2017) there was an increase in CD86 and T-Cell CD8 dendritic cells and a decrease in CD45 and TAM penetration in this study with targeted deletion of the RELAsubunit. Because NF-κB is a convergent point for numerous intercellular pathways, knocking down the RELA subunit in combination with anti-inflammatory medicines could be used to target different molecular mechanisms and components in the TME to prevent cancer-stimulating inflammation

Natural products, according to clinical studies, are a powerful anti-cancer therapy that can treat tumors and reverse multidrug resistance (Zhang et al., 2017). For this purpose, the study of molecular docking using computational techniques on the RELA gene to corroborate the NF-κB inflammatory pathway and the importance of the involvement of the RELA subunit in the advancement of GBM .cancer was performed In order to, introduce a candidate medicine with better performance and potential as a combination therapy to improve the effectiveness of the present study.

Based on the results of earlier investigations, Ecteinascidin-743, Bortezomib, Chromomycin A3 and digitoxin were chosen for molecular docking. According to pre-clinical research, the anti-cancer efficacy of Ecteinascidin-743 is attributed to anti-inflammatory effects by modifying the production of selective cytokines by inhibiting NF-κB signaling as well as reducing the number and penetration of cells TAM through apoptosis induction with anti-angiogenesis effects (Germano et al., 2013, 2010).

Because of Trabectedin's efficacy, various studies have looked into the possibility of combining it with other inhibitors and radiation therapy, all of which indicate Trabectedin's function in inflammatory-related tumors by reducing ,.possible growth-related variables (Miao et al Zhu et al., 2019). pre-clinical studies show ;2016 that Bortezomib, a proteasome inhibitor, has not yet been considered a single-agent treatment for GBM due to its toxic effects on various tumour cells. It has, however, been introduced to improve cell sensitivity in combination therapy with other anti-cancer medications (Su et al., 2021; Tan et al., 2019). Digitoxin, as a cardiac glycoside, possesses anti-cancer properties, possibly by blocking HIF-1α production in cancer cells. Recent research has found that this medication has anti-inflammatory properties and could be used to treat cancer and inflammatory illnesses by blocking the TNFα/NF-κB signalling pathway and the production of proinflammatory cytokines (Kumavath et al., 2021). Recently demonstrated, Chromomycin A3 drug- a natural antibiotic with anti-cancer characteristics- has a dose-dependent mechanism of action. The medication was found as an NF-κB inhibitor with a similar function to Ecteinascidin-743 (Miller et al., 2010; Saranaruk et al., 2020).

According to docking data from earlier studies, any of the medicines can be employed as an NF-κB inhibitor. However, of the four bioactive chemicals chosen, Ectinascidin-743 had the lowest binding energy while also having the best effect when compared to the other three medications. As a result of the findings of the preceding investigations, Ectinascidin-743 can be considered as a possible candidate for inhibiting NF-κB to improve therapy sensitivity and efficacy.

It is suggested that this study be performed on other cell lines, signalling pathways and NF-κB-RELA ted stem genes. It is proposed that the Western method be used to confirm the final results obtained at the protein level. Functional studies are also needed to examine the exact role of genes and drug efficacy.

CONCLUSION

In conclusion, the RELA subunit seems to be one of the factors affecting hypoxia, apoptosis, and change in stemness genes expression levels in GBM. Therefore, it is recommended to knock out the NF-κB signaling pathway or to use Ecteinascidin-743 in future studies.

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Declaration of interest statement

The authors declare no potential conflicts of interest to disclose.

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Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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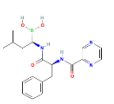
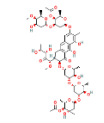
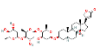
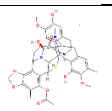
TABLE 1. The primers used for Real-Time PCR.

Name	Forward sequence (5 ′-3 ′)	Reverse sequence (5 ′-3 ′)
HIF-1α	TAGAAAGCAGTTCCGCAAGC	TGGTGGCATTAGCAGTAGGT
NES	CACCCCTCAGCCCTGACCACT	CCCTCTATGGCTGTTTCTTTCTCT ACCA
GAPDH	CTCTCTGCTCCTCCTGTTCG	ACGACCAAATCCGTTGACTC

TABLE 2. The docking results obtained by different drug candidates in interaction with the target protein.

	Structure	PubChem ID	Chemical formula	Binding Affinity
Bortezomib		387447	C19H25BN4O4	-7.3
Chromomycin A3		656673	C57H82O26	-7.8
Digitoxin		441207	C41H64O13	-9.2
Ectinascidin-743		372978	C39H43N3O11S	-14.3

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