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SYNERGISTIC EFFECT OF CURCUMIN AND ATIPRIMOD AS POTENT INHIBITORS OF STAT3 AND IL-6 RECEPTOR IN CHORDOMA CELLS

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Abstract

Chordoma, is a rare bone tumor, which is characterized by a high recurrence rate and drug resistance in addition to its potential for local invasion, and metastasis. It is a low-grade axial skeletal carcinoma derived from notochord remnants. Molecular pathways that underlie the mechanisms of chordoma pathogenesis are partially elucidated, however, the rate of success in treatment remains to be solved. Constitutively active STAT3 and partially active STAT5 suppress anti-tumor immunity, resulting in increased proliferation, survival and aggressiveness of tumor cells. Persistent activation of STAT3 mediates tumor-promoting inflammation. STAT3 upregulates pro-oncogenic inflammatory pathways, including nuclear factor-κB (NFκB), interleukin-6 (IL-6), and Janus kinase (JAK) pathways. In conclusion, IL6R and STAT3 are promising targets for rerouting inflammation for cancer therapy. In this study, curcumin and atiprimod agents were applied to chordoma cell lines in combination based on molecular docking analyses. The binding efficacy was found favorable for the treatment with two agents and synergistic anti-cancer effects of this combined application were detected on chordoma cells. Molecular docking analyses together with the in vitro results support the idea that application of IL-6R and Stat3 co-inhibition have lethal effects on chordoma cells.

Keywords: IL-6R, Atiprimod, Curcumin, Molecular Docking, Chordoma

KORDOMA HÜCRELERİNDE STAT3 VE IL-6 RESEPTÖRÜNÜN GÜÇLÜ İNHİBİTÖRLERİ OLARAK KURKUMİN VE ATİPRİMODUN SİNERJİK ETKİSİ

Özet

Kordoma, lokal invazyon ve metastaz potansiyelinin yanı sıra yüksek nüks oranı ve ilaç direnci ile karakterize, nadir görülen bir kemik tümörüdür. Notokord kalıntılarından kaynaklanan düşük dereceli aksiyal iskelet karsinomudur. Kordoma patogenez mekanizmalarının altında yatan moleküler yolaklar kısmen aydınlatılmış olmakla birlikte tedavideki başarı oranı henüz çözülmemiştir. Yapısal olarak aktif STAT3 ve kısmen aktif STAT5, anti-tümör bağışıklığını baskılayarak tümör hücrelerinin çoğalmasının, hayatta kalmasının ve saldırganlığının artmasına neden olur. STAT3'ün kalıcı aktivasyonu, tümörü teşvik eden inflamasyona aracılık eder. STAT3, nükleer faktör-κB (NFκB), interlökin-6 (IL-6), ve Janus kinaz (JAK) yolakları dahil olmak üzere pro-onkojenik inflamatuar yolakları düzenler. Sonuç olarak IL6R ve STAT3, kanser tedavisi için inflamasyonun yeniden yönlendirilmesi açısından umut verici bir hedeftir. Bu çalışmada, moleküler yerleştirme analizleri sonucunda kordoma hücre hatlarına kurkumin ve atiprimod ajanları kombinasyon halinde uygulanmıştır. İki ajanın tedavisi için bağlanma etkinliği olumlu, bu kombine uygulamanın sinerjistik antikanser etkilerinin olduğu bulunmuştur. Moleküler yerleştirme ve in vitro sonuçlar, IL-6R ve Stat3 ortak inhibisyonunun kordoma hücreleri üzerinde öldürücü etkileri olduğu fikrini desteklemektedir.

Anahtar Kelimeler: IL-6R, Atiprimod, Kurkumin, Moleküler Yerleştirme, Kordoma

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1. Introduction

Chordoma is a rare tumor that accounts for 1-4% of all bone malignancies. Histologically, these tumors usually exhibit low-level altered but clinically malignant behavior characterized by local invasion. Clinically, chordomas are locally aggressive and have a high incidence for recurrence similar with other malignant tumors [1]. Chordomas derive from undifferentiated notochord remnants present throughout the axial skeleton. As a result, these tumors can occur in the skull base, mobile spine, and sacrum. The coincidence is evenly distributed in each of these regions [1]. Chordoma, which occurs at the base of the skull, is particularly problematic due to its proximity to critical

bone, vascular, and nerve structures. This feature significantly compromises the ability to surgically resect the entire tumor tissue, which is the mainstay of early tumor therapy. The aim of surgical treatment is maximum resection performed in the context of neuroprotection. Failure to achieve complete resection results in recurrence rates approximately four times higher than in cases with ideal complete resection [2]. Although whole tissue resection is achievable in less than 50% of skull base chordomas [1], regardless of whether complete resection is performed, the recurrence rates remain at significant rates and endanger the patient's life. Therefore, there is a greater need for conventional chemotherapy, which is one of the most common treatment methods used for patients who have undergone total resection for the treatment of chordoma or whose treatment is very dangerous. Unfortunately, because chordoma cells have a poor response to chemotherapy, scientists have sought many alternative agents to supplement treatment.

Signal transducer and activator of transcription (STAT) proteins are a family of cytoplasmic transcription factors that share a general structure, organized into functional modular domains. The STAT family mediates multiple intracellular signaling pathways [3]. Among these, STAT3 is involved in numerous biological processes such as cell proliferation, survival, differentiation, and angiogenesis [4,5]. In normal cells, transient activation of STAT3 transmits transcriptional signals from cytokines and growth factor receptors on the plasma membrane to the nucleus [3]. In contrast, STAT3 shows high activity in most human cancers and is generally associated with a poor clinical prognosis [6]. Therefore, the STAT3 signaling pathway has long been recognized as a potential therapeutic target for cancer therapy due to its roles in tumorigenesis, metastasis, and drug resistance [7-10]. Various studies indicated that inhibiton of IL-6 suppressed the tumorigenicity of chordoma cells [11].

Molecular docking provides new insights into increasing the efficiency of drug trials in many cancer types. The method is based on protein-ligand interaction, which may enable the binding of drugs to the cells prior as wells as post application. One can employ this technique at many phases of drug design and drug repurposing techniques, for example, to simplify the creation of promising drug candidates [12,13]. It is crucial to identify the optimum ligand poses and rate the relative docking propensities of various ligands. A 3D structure of potential ligands and the protein of interest is required for molecular docking to be performed, as well as a method to determine the poses and intensities of interactions between the two. The development of combinatorial chemistry led the number of synthetic chemical libraries becoming rapidly more plentiful, which raised the need for quick and inexpensive methods to investigate interactions of target proteins [14]. The frequent use of docking techniques as an essential step in virtual screening is due in large part to the growing quantity of Protein Data Bank (PDB) and chemical library data, as well as the strong need to anticipate binding modes as well as binding affinities of ligands [15]. There are several molecular docking methods available for prediction of protein-ligand interactions and evaluation, which requires use of scoring systems unique to the docking method [16,17]. Docking software uses protein-ligand sampling approaches to give adequate ligand poses for the smoothness of data acquired in subsequent drug repositioning research that enables researchers to investigate the risks of unfavorable side effects and medication interactions during drug development. The computational or in silico procedures are quick and offers fascinating contribution to the field of drug repurposing [18]. The massive increase in computing power, accessibility, and the growing availability of small chemical and protein libraries have all substantially aided this process [19-21].

2. Methods

2.1. Experimental Design

We aim to divide this study into two parts in which the first part comprises of in silico screening and the latter is in vitro validation and conducting functional assays. We performed a computational molecular docking modelling and specified two compounds as STAT3 and IL-6 receptor inhibitors. The second part investigates the binding affinities between the compounds and the target genes. Based on the in silico binding of the drugs to the target genes, in vitro assays including cell cycle, viability, and gene expression analyses were performed on two chordoma cell line models. Upon induction by the agents in combination and/or alone will be suggested as a novel therapeutic approach in chordoma treatment.

2.2. Molecular Docking Analyses

After the amino acid sequences of BAK, MUC1 and CASPASE 3 proteins were obtained from NCBI for the molecular docking process, the 3D structures of the proteins were estimated using I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) [22]. The predicted protein models were then refined using ModRefiner (https://zhanggroup.org/ModRefiner/) [23]. Refined protein models were visualized and structural variations were mapped by UCSF Chimera 1.14 [24]. For the 3D models of IL-6 and BAK proteins, data from the Protein Data Bank (PDB) were retrieved. 3D models for Atiprimod and Curcumin (Figure 1a and b, respectively) were obtained from DrugBank. After saving all protein models and ligands in pdb file format, molecular docking was performed on 5 different proteins using the CB-Dock server (https://cadd.labshare.cn/cb-dock2/) [25]. IL-6 inhibitors curcumin and atiprimod were used for molecular docking, which was analyzed through 5 different cavities.

2.3. In vitro Analyses

Chordoma cell lines CH22 and MUG-Chor1 were kindly obtained from Chordoma Foundation and grown in the following culture conditions. The cells were cultured in media containing Iscove's Modified Dulbecco's Medium (IMDM) (31980030, Invitrogen, UK) and RPMI 1640 (11875093, Gibco,

Invitrogen, UK) in 4:1 ratio, respectively, supplemented with 10% Fetal Bovine Serum (FBS) (10500-064, Invitrogen, Gibco, UK) and 1% Antibiotic-Antimycotic (15240096, Gibco, UK) and incubated in a humidified 5% CO2 37◦C chamber.

2.4. Viability Assay

Cell viability assay was conducted by 3-(4,5-dimenthylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTS) method. Chordoma cells were seeded at 3 × 10^3/ml in 96-well plates and incubated for overnight. The next day, cells were treated with the agents alone and in combination at defined concentrations. Viability assay was performed upon 72-hour treatment. Briefly, the agents were removed and the MTS solution was applied onto each well of treatment for one hour until the color change observed. The absorbance values were detected by the ELISA (ELx800, Biotek Instruments, USA) plate reader at 490 nm wavelength and the values were normalized to the ones obtained from untreated control cells.

2.5. Statistical Analysis

For the statistical analysis, the viability assay was carried out in triplicates and GraphPad Prism 7.0 program was used for multiple comparisons among atiprimod and curcumin treated MUG-Chor1 and CH-22 cells with their untreated counterparts. One-way analysis of variance (ANOVA) with Tukey's post-hoc test was applied to data to determine the significance of differences.

3. Methods

3.1 In silico Approach

I-TASSER was used to predict the 3D structures of the proteins Bak, Bax, Casp3, Muc1, Stat3, and IL6, and the structures were further refined by ModRefiner as demonstrated in Figure 1. In Figure 2, the 2D structures of binding regions of atiprimod on each protein were depicted. According to the Vino scores shown in Table 1, the potential binding for atiprimod to the proteins were found to be -6.5, -7.5, -6, -6.5, -6.9, -6.8, and -7, respectively. Figure 3 shows 2D structures of binding regions of Curcumin on each protein. Overall, curcumin was found to have the stronger binding efficacy over atiprimod on all proteins. Specifically, the binding scores for each protein was found to be -7.1, -7.7, -7.4, -6.9, -7.6, -7.3, and -8, respectively.

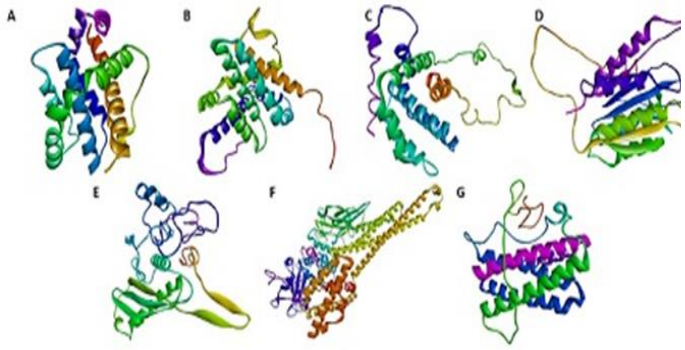

Figure 1. 3D Structures of proteins: A: BAK, B: BAX, C: BCL2, D: CASP3, E: MUC1, F: STAT3, G: IL-6 receptors.

Table 1. VINO scores of each molecule against the molecular targets.

	Drugs	
	Atiprimod	Curcumin
BAK	-6,5	-7,1
BAX	-7,5	-7,7
BCL2	-6	-7,4
CASP3	-6,5	-6,9
MUC1	-6,9	-7,6
STAT3	-6,8	-7,3
IL-6	-7	-8

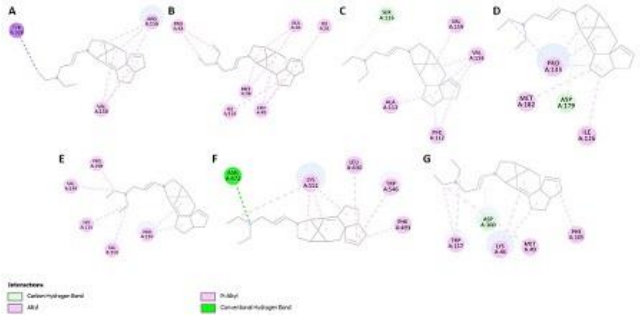

Figure 2. The Binding regions for Atiprimod on each protein: A: BAK, B: BAX, C: BCL2, D: CASP3, E: MUC1, F: STAT3, G: IL-6R

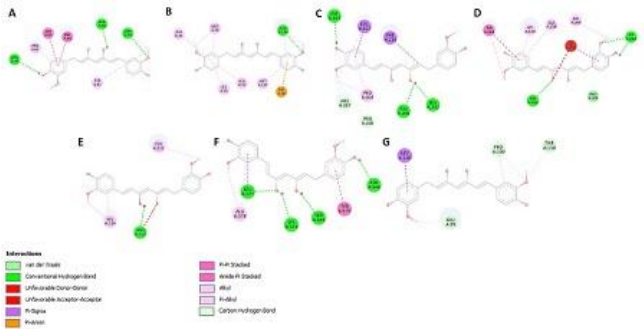

Figure 3. The Binding regions for Curcumin on each protein: A: BAK, B: BAX, C: BCL2, D: CASP3, E: MUC1, F: STAT3, G: IL-6R

3.2. In vitro Studies

The chordoma cells are negatively impacted by the combined therapy. Viability studies revealed that chordoma cells treated with a combination of Atiprimod and Curcumin were more lethal than those treated with each drug alone. As seen in Figure 4A, atiprimod reduced the vitality of CH-22 cells by less than 50%, but curcumin alone had no lethal impact on the cells. The vitality of CH-22 cells decreased significantly more when they were combined, indicating a synergistic impact that increased toxicity. Similar to this, MUG-Chor1 cells treated with

both medications experienced a greater death rate compared to those treated with only one drug (Fig. 4B). In general, combinatorial treatment had better effect on CH-22 cells than MUG-Chor1 cells.

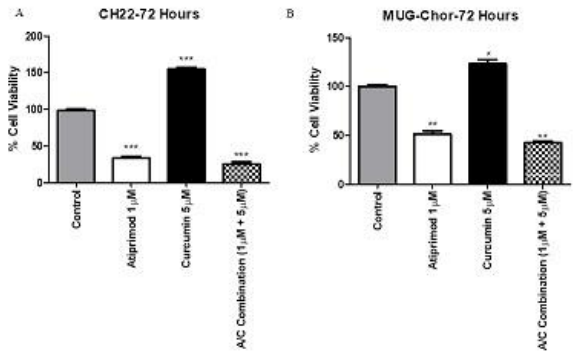

Figure 4. (A) The viability percentage of CH22 cells treated with Curcumin, Atiprimod and combination of the two drugs. (B) The viability percentage of MUG-Chor1 cells treated with Curcumin, Atiprimod and combination of the two drugs. P<0,05

4.Discussion

STAT3 becomes overactive in many human cancers and serves as a crucial signaling pathway for tumor cells and the tumor microenvironment of cells, particularly tumor infiltrating immune cells. Therefore, targeting STAT3 is expected to reduce enhanced anti-tumor effects of tumor-infiltrating immune cells, increase immunosuppressive factors in the tumor microenvironment, and thus increase tumor cell proliferation [26]. These effects position STAT3 as an emerging potential promising target for cancer therapy. STAT3, a member of the STAT family, has been identified as a common target that controls the signaling of proinflammatory cytokines and growth factors, as well as various signaling pathways that regulate oncogenes [27]. This factor contributes to the growth and survival of the cell and increases the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, thereby blocking apoptosis. Furthermore, STAT3 is known to be the molecular target of curcumin through direct and/or indirect inhibition of IL-6 receptor [28-29] in various tumors.

Previous studies indicate that curcumin reduces STAT3 activity and used in the treatment of a variety of tumor cells [30]. According to a study done by Guan et al., treatment with curcumin may induce autophagy-mediated death by degrading AKT-also known as protein kinase B, which is associated with carcinogenesis- in breast cancer cells [31]. Mammalian target of Rapamycin (MTOR) plays a role in the control of cancer cell growth and proliferation and also induces autophagy by suppressing the ubiquitin-proteasome pathway [32]. In addition, curcumin has been hypothesized to support apoptotic and autophagy by blocking the PI3K/AKT signaling pathway in breast cancer cells [32]. In another study, apoptotic effect induced by curcumin agent was observed after treatment of MCF-7 cells with curcumin and PI3K inhibitor. A synergistic effect of curcumin and PI3K inhibitor has been demonstrated in breast cancer [33]. Curcumin interferes with the EGFR signaling pathway, a family of receptor tyrosine kinases reported to be associated with proliferation, adhesion [34], migration and differentiation of cancer cells [32]. Therefore, modulation of EGFR may represent a good strategy for cancer therapy. Curcumin inhibited the growth and proliferation of breast cancer cells by reducing EGFR [35-37], and AKT levels [34,38].

The curcumin agent has been observed to inhibit JAK2 activity by downregulating the expression of NF-κβ in the A549 cell line and via the JAK2/STAT3 signaling pathway [30]. Curcumin induced apoptosis of non-small cell lung cancer cells [38] through upregulation of microRNA-192-5p and suppression of the PI3K/AKT signaling pathway [39]. A novel cationic lipid nanosystem containing curcumin has been reported to exhibit better cytotoxicity, increase antiproliferative, proapoptotic and anti-invasive activities, and arrest cell cycle in Lewis lung cancer cells [40]. Curcumin induced G0/G1 phase arrest through MTA1-mediated inactivation of the Wnt/β-catenin pathway in non-small cell lung cancer and affected cellular progression [41]. However in a study done by Dance-Barnes, it was surprising to see that curcumin accelerated the development of lung lesions from benign hyperplasias to adenomas and carcinomas [42]. This confirmed our findings that depending on the type of tumor, curcumin may exert cancer promotion effects as well. Although, it is established that the curcumin agent suppresses tumors of various kinds of tumors, the impact of the curcumin compound on chordoma was examined in only one study. Using the CM-319 cell line, this study examined the apoptotic properties of curcumin and its susceptibility to radiation [43].

Atiprimod is a new orally bioavailable cationic amphiphilic agent that has been investigated for its anti-inflammatory and anti-cancer properties. It has been shown in many types of cancer that atiprimod supresses the STAT3 gene activity and inhibits the proliferation of cancer cells. Recent studies suggest that the atiprimod exhibits antiproliferative and antiangiogenic activities, induces apoptosis through caspase-3 and caspase-9 dependent activation, decreases IL-6 [44] and VEGF production, and inhibits phosphorylation of protein kinase B and STAT3 [45]. Early clinical observations have recently revealed significant tumor regression in a few patients with advanced liver carcinoid cancer, along with reduction of other debilitating symptoms [36]. Despite these extensive preclinical and clinical studies, the mechanism of action of atiprimod needs to be clarified.

Faderl et al. showed that atiprimod inhibited the clonogenic growth of acute myeloid leukemia cells and newly isolated acute myeloid leukemia marrow cells [46]. The anti-proliferative and pro-apoptotic activities

of atiprimod in acute myeloid leukemia cells are thought to affect the activity of the JAK-STAT pathway, a receptor-independent signaling pathway whose extracellular stimuli control gene expression [47].

JAK2, a member of the transcription factors family, is known as the main downstream effectors of STAT proteins, so negative regulation of JAK2 would be expected to result in negative regulation of STAT proteins. Atiprimod inhibits STAT-3 and STAT-5 phosphorylation, which may be due to a decrease in JAK2 levels (protein or gene expression or phosphorylation). Constitutive activation of STATs is known to be expressed in leukemia cells as well as from patients with acute myeloid leukemia [48]. Some hematopoietic cytokines are known to play a role in the stimulation of the JAK/STAT pathway. Therefore, it is possible that these hematopoietic cytokines are involved in STAT pathway activation. For example, the overexpression of IL6 receptors in leukemia patients is known to cause constitutive STAT-3 activity[6].

5.Conclusion

Treatment options for chordoma, a rare kind of bone tumor, are moderately limited. Only a small number of the several pathways implicated in chordoma pathogenesis that were addressed showed some degree of success. Using in silico techniques, a combination of several molecules may be able to eradicate tumor-causing cells in vitro. This allows one to repurpose medications and small molecules and determine which is the most effective way to target tumor cells. Two well-known substances were chosen for this investigation based on their ability to bind to proteins—which are abundant on the surfaces of chordoma cells—in the most effective manner. The molecular docking analysis indicated that when cells were treated with both atiprimod and curcumin together, IL-6 receptor was the most favorable target for binding. The two substances' ability to kill chordoma cells is further supported by in vitro tests. For the first time, we demonstrated that the combined effects of atiprimod and curcumin on the viability of chordoma cells enhanced and yet further investigation is required.

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7. References

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