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Research Article

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The Role of Aryl Hydrocarbon Receptor in Proliferation and EMT Induction in Neuroblastoma Cancer Cells (SH-SY5Y)

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Abstract

Neuroblastoma is a common and aggressive cancer in children characterized by metastasis and uncontrolled cell proliferation. The Aryl Hydrocarbon Receptor (AhR) is a crucial regulator of diverse biological processes, such as cell growth, immune responses, and metabolism. However, its role in neuroblastoma progression is inadequately investigated. SH-SY5Y neuroblastoma cells were treated with the AhR antagonist CH223191, the agonist benzo[a]pyrene (BaP), and the endogenous ligand 6-Formylindolo[3,2-b]carbazole (FICZ). Cell viability was evaluated by MTT assay, and ROS levels were measured by dichlorofluorescein (DCF) assay, while EMT-related gene expression (*vimentin* and *E-cadherin*) was analyzed using qPCR. Compared to control, treatment with CH223191 and BaP significantly decreased cell viability and increased ROS production, respectively. BaP induced a greater reduction in viability and increase in ROS than FICZ ($p < 0.01$). Gene expression analysis revealed that CH223191 and BaP decreased *vimentin* expression ($p < 0.05$ and $p < 0.001$) and increased *E-cadherin* expression ($p < 0.05$ and $p < 0.001$), indicating the inhibition of EMT and promotion of epithelial characteristics. These findings highlight the pivotal role of AhR in modulating proliferation, mitochondrial function, and EMT in neuroblastoma cells, indicating that targeting AhR signaling may offer a novel therapeutic strategy to impede neuroblastoma progression and metastasis.

Keywords: Neuroblastoma; Aryl Hydrocarbon Receptor; Proliferation; ROS; EMT.

1. Introduction

Neuroblastoma is the most common extracranial solid malignancy in children [1]. Although it has a low incidence of 6–11 cases per million children annually worldwide, neuroblastoma constitutes ~15% of all pediatric cancer-related fatalities [2-4]. The disease demonstrates significant clinical heterogeneity, exhibiting a spectrum from spontaneous regression to aggressive progression resistant to multimodal therapy [5]. This variability is due to a complex interplay of

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genetic factors, including MYCN amplification, epigenetic alterations, and the effects of the tumor microenvironment, which collectively influence tumor behavior and treatment response [4, 6].

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates xenobiotic metabolism [7, 8]. However, it is increasingly evident that AhR transcends xenobiotic detoxification and has a prominent role in developmental processes, immune regulation, cell cycle control, and differentiation [9]. AhR signaling dysregulation has been implicated in various cancers, including neuroblastoma, where its role appears to be context-dependent. Indeed, AhR has been linked to both oncogenic and tumor-suppressive functions in neuroblastoma. For example, in non–MYCN–amplified disease, high AhR expression levels correlate with reduced proliferation, which is partly mediated by endogenous activators of AhR, such as kynurenine [4]. Conversely, in MYCN-amplified neuroblastoma, AhR facilitates cancer cell growth and inhibits retinoic acid–induced differentiation, partially via epigenetic processes [10]. Pharmacological modulation of AhR is a prospective therapeutic approach, as agonists, antagonists, and selective modulators exhibit the potential to influence neuroblastoma cell fate [11].

A key regulator of neuroblastoma aggressiveness is the epithelial–mesenchymal transition (EMT), a process that enhances the migratory and metastatic potential of cancer cells [12]. EMT in neuroblastoma can be induced by diverse signaling pathways such as TGF-β1/Gli, leading to poor prognosis [13]. Importantly, recent research has revealed that AhR may interact with EMT regulation in a ligand-specific manner: in epithelial malignancies, AhR activation often promotes EMT, whereas in other contexts, it may stabilize epithelial characteristics and inhibit invasion [14].

Building on the utility of the SH-SY5Y cell line as a well-established in vitro model that recapitulates essential aspects of neuroblastoma biology and differentiation, and considering the dual, context-dependent role of AhR signaling and its potential crosstalk with EMT pathways, the present study aims to elucidate the functional consequences of AhR modulation in neuroblastoma.

2. Materials and Methods

2.1. Reagents

6-formylindolo [3,2-b] carbazole (FICZ) and Benzo[a]pyrene, >96% purity (BaP, B1760), and pure AhR antagonist (CH-223191, >99% purity, Cat. No.: A8609), were obtained from Sigma-Aldrich and ApexBio Technology, respectively. All chemicals were dissolved in Dimethyl sulfoxide (DMSO, Sigma-Aldrich) as a stock solution. The DMSO final concentration in experiments was <0.1%.

2.2. Cell Culture

The SH-SY5Y cells were obtained from the Iranian Biological Resource Center. Cells were cultured in high-glucose DMEM (Biowest, France) supplemented with 10% FBS (Biowest, France) and 1% penicillin–streptomycin solution (Biowest, France). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Cell Viability Assay

Cell viability was assessed using the MTT assay. SH-SY5Y cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 hours, cells were treated with FICZ (10 nM), CH-223191 (100 nM), or BaP (2.5 µM) for 48 hours. Then, the culture medium was replaced with 100 µL of medium containing 5 mg/mL MTT and incubated for another 4 hours to allow for formazan crystal formation. Finally, the formazan crystals were solubilized in DMSO, and absorbance was measured at 570 nm using a microplate reader.

2.4. Reactive Oxygen Species (ROS) Measurement

Cells were seeded in 96-well plates at 1×10^4 cells/well and treated as described above (n = 3). After washing, cells were incubated with 100 µL of 26 µM 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 30 minutes, after which fluorescence of the oxidized product (DCF) was measured at 485/520 nm. Following the removal of the medium, cells were lysed by the addition of 100 µL of 0.5 M sodium hydroxide. After gently mixing for 30 minutes, the total protein content was assessed by adding 100 µL of Bradford reagent. All measurements were subsequently normalized to the corresponding protein concentrations.

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2.5. Quantitative Real-Time PCR (qRT-PCR)

SH-SY5Y cells were seeded in 6-well plates at a density of 5 × 10³ cells/well and treated as described in the MTT assay for 48 hours. Next, using Trizol reagent (Bio Basic, Canada), total RNA was extracted, and then cDNA was synthesized using a reverse transcription kit (Bio Basic, Canada). qRT-PCR was performed using SYBR Green Master Mix (Ampliqon, Denmark) on an ABI StepOnePlus real-time PCR system. The GAPDH gene was used as the housekeeping control. The 2^(-ΔΔCt) method is used for relative gene expression. Primer sequences are as follows (**Table 1**):

2.6. Statistical Analysis

Data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test, conducted in GraphPad Prism version 8. p-values less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. The effects of AhR ligands on SH-SY5Y cell viability

The viability of cancer cells treated with CH223191 and BaP after 24 (**Figure 1A**) and 48 h (**Figure 1B**) was significantly decreased compared to the control group ($p < 0.05$ and $p < 0.001$, respectively). Furthermore, the viability of cancer cells treated with BaP was significantly lower than that of cells treated with FICZ ($p < 0.001$). The effects of different AhR ligands on SH-SY5Y cell morphology are consistent with cytotoxicity results (**Figure 1C**).

Table 1. The Primer sequences

Gene	Forward Primer	Reverse Primer
GAPDH	5'-GAAGGTGAAGGTCGGAGTCAAC-3'	5'-CAGAGTTAAAAG CAGCCCTGGT-3'
Vimentin	5'-TCTACGAGGAGGAGATGCGG-3'	5-GGTCAAGACGTGCCAGAGAC-3
E-Cadherin	5'-CAGGAGTCATCAGTGTGGT-3'	5'-GGAGGATTATCGTTGGTGTCAG-3'

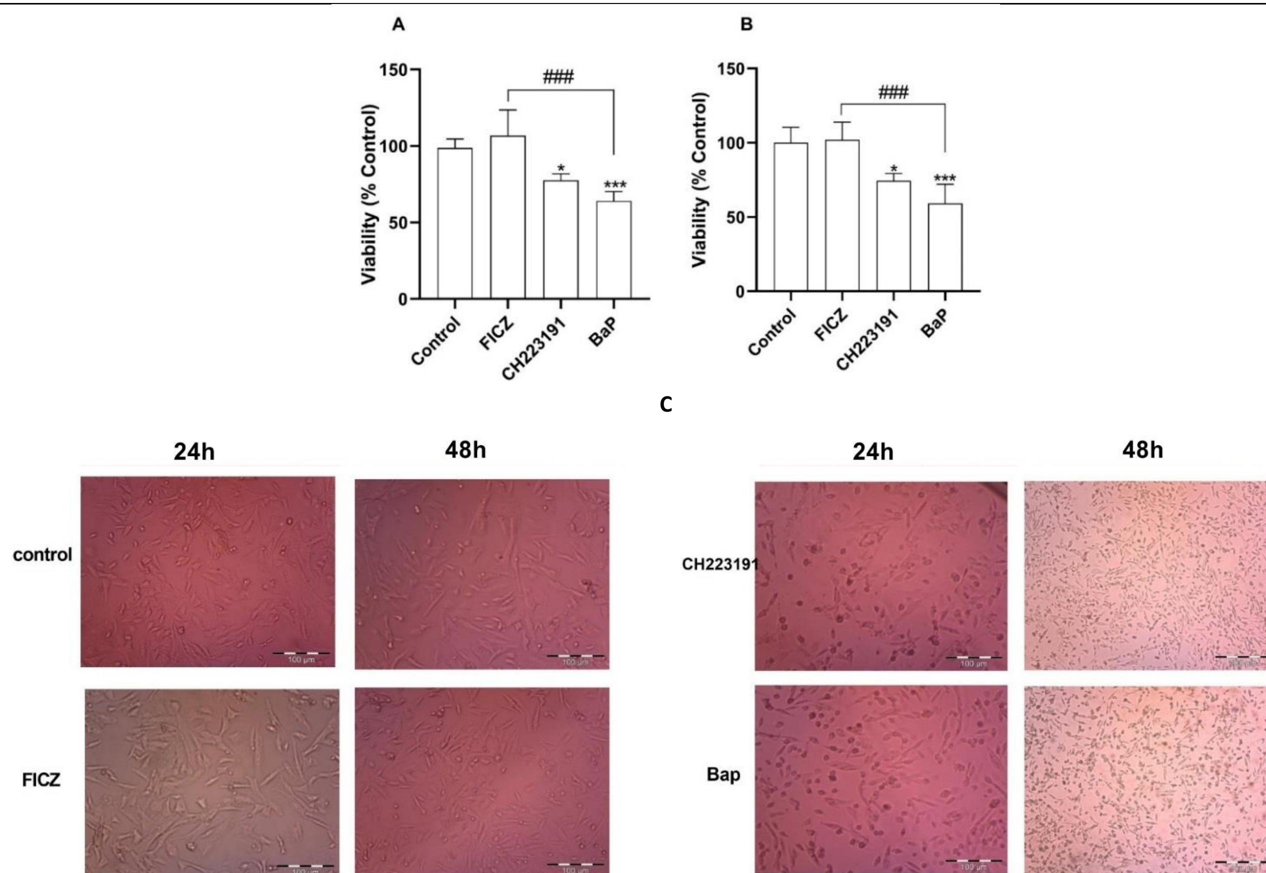

Figure 1. The effect of exposure to AhR ligands on cell viability in SH-SY5Y cells was assessed using the MTT assay after 24 hours (A) and 48 hours (B), and cell morphology. Three independent experiments were conducted, each with eight replicates. Asterisks indicate statistically significant differences compared to the control group (*$p < 0.05$, ***$p < 0.001$), while the hash symbol (###) indicates a significant difference ($p < 0.001$) compared to the FICZ-treated group.

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3.2. The effects of AhR ligands on ROS production in SH-SY5Y cells

Treatment with CH223191 and BaP significantly increased ROS production. As shown in **Figure 2A**, compared to the control group, ROS levels were significantly enhanced after treatment with CH223191 and BaP ($p < 0.05$ and $p < 0.01$, respectively). Additionally, BaP-treated cells exhibited higher ROS levels than those treated with FICZ ($p < 0.05$). This trend was further confirmed in **Figure 2B**, where CH223191 and BaP again significantly increased ROS production relative to control ($p < 0.01$ and $p < 0.001$, respectively), and BaP induced significantly greater ROS levels than FICZ ($p < 0.01$). These findings indicate a consistent and time-dependent pro-oxidant effect of BaP and CH223191 on SH-SY5Y cells.

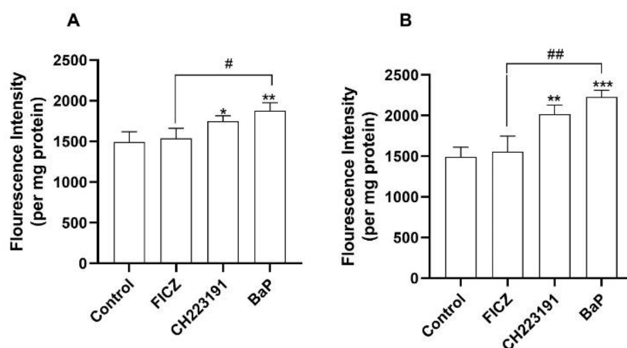

Figure 2. The effect of exposure to AhR ligands on ROS production in SH-SY5Y cells after 24 hours (A) and 48 hours (B). We conducted three independent experiments, each consisting of eight replicates. Asterisks indicate statistically significant differences compared to the control group (*$p < 0.05$, (**$p < 0.01$, ***$p < 0.001$), while the hash symbol (#$p < 0.05$, ## $p < 0.01$) indicates a significant difference compared to the FICZ-treated group.

3.3. The effects of AhR ligands on EMT gene expression in SH-SY5Y cells

Figure 3 presents the effects of CH223191 and BaP on the expression of two key EMT markers in cancer cells. Treatment with CH223191 and BaP significantly reduced *vimentin* gene expression compared to the control (**Figure 3A**, $p < 0.05$ and $p < 0.001$, respectively). Furthermore, *vimentin* expression in BaP-treated cells was significantly lower than in those treated with FICZ and CH223191 ($p < 0.001$). In contrast, **Figure 3B** indicated that *E-cadherin* expression was significantly upregulated in the CH223191 and BaP-treated groups compared to the control ($p < 0.05$ and $p < 0.001$, respectively). Notably, BaP-treated cells exhibited significantly higher *E-cadherin* expression compared to cells treated with FICZ ($p < 0.001$) and CH223191 ($p < 0.01$). These results suggest that BaP and CH223191 may suppress EMT by downregulating mesenchymal markers while upregulating epithelial markers.

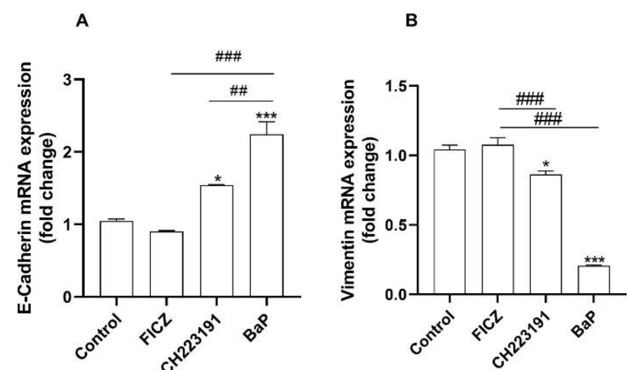

Figure 3. The effect of exposure to AhR ligands on *E-Cadherin* (A) and *Vimentin* (B) gene expression in SH-SY5Y cells after 48 hours. We conducted three independent experiments. Asterisks indicate statistically significant differences compared to the control group (*$p < 0.05$, ***$p < 0.001$), while the hash symbol (##$p < 0.01$, ### $p < 0.001$) indicates a significant difference compared to the FICZ-treated group.

3.4. Discussion

This study demonstrates that the AhR ligands BaP, FICZ, and CH-223191 differentially modulate cell viability, ROS production, and EMT markers expression in SH-SY5Y neuroblastoma cells. In addition, the distinct effects of AhR agonists (such as BaP and FICZ) and the antagonist CH223191 on these cellular processes suggest that AhR modulation plays a crucial role in neuroblastoma pathogenesis, providing potential therapeutic targets. Recent studies have emphasized the dual roles of AhR in cancer biology, where AhR agonists can either promote or inhibit tumor progression, based on the nature of the ligand, its concentration, and the specific cancer context [15].

Our results indicated that CH223191 and BaP significantly decreased the viability of SH-SY5Y cells,

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with BaP inducing a more profound effect than FICZ. Interestingly, despite its potency as an endogenous AhR agonist, FICZ induced a less marked decrease in cell viability than BaP. This disparity likely arises from differences in the specific cellular signaling pathways and transcriptional programs activated by each ligand. AhR activation by dioxin-like compounds is distinguished by the prolonged induction of cytochrome P450 enzymes and other metabolizing enzymes, whereas other agonists typically elicit only transient enzyme induction. These findings show that endogenous AhR ligands are probably rapidly metabolized to facilitate regulatory control [16]. While both FICZ and BaP activate AhR signaling, FICZ is quickly metabolized by CYP1A1 into less active derivatives, resulting in a negative feedback loop that limits sustained [16, 17]. In contrast, BaP metabolism produces toxic intermediates, such as quinones and epoxides, that induce mitochondrial impairment, oxidative stress, and apoptosis [18]. These results support the concept of ligand-selective AhR modulation, in which various ligands elicit varied transcriptional outcomes, with endogenous ligands such as FICZ maintaining physiological balance and xenobiotics like BaP triggering cellular damage [19].

Increased ROS levels following BaP or CH223191 treatment, indicating mitochondrial involvement in cytotoxicity. Interestingly, the cytotoxicity of CH223191 is consistent with recent results showing that AhR antagonism can alter pro-survival pathways and decrease cancer cell proliferation [20, 21]. This suggests that both activation and inhibition of AhR may converge on mitochondrial stress pathways in SH-SY5Y cells, albeit via different mechanisms.

Our results also revealed that both BaP and CH223191 significantly altered EMT-associated gene expression, resulting in the upregulation of *E-cadherin* and the downregulation of *vimentin*. This pattern implies that EMT-like processes are suppressed in SH-SY5Y cells. In neuroblastoma, which has both neuronal and epithelial characteristics, such a shift may indicate a reduction in migratory potential. These findings are somewhat unexpected, considering that AhR activation has been associated with EMT promotion in different epithelial malignancies, including lung and breast carcinoma [22, 23]. When compared with other studies, our results highlight the importance of cellular context. Our findings indicate that both AhR activation and inhibition can shift the balance between epithelial and neuroblast characteristics in SH-SY5Y cells, with EMT markers responding differently than in epithelial cancers, thereby promoting a more epithelial-like cellular phenotype. This discrepancy may be attributed to differences in basal AhR expression, cofactor availability, and CYP1A1 activity between neural cells and epithelial cancers. Indeed, previous work has demonstrated that neuroblastoma cells express functional AhR, and its activity is linked to differentiation status and MYCN regulation [24]. As a result, the opposing effects of AhR modulation in neuroblastoma versus other malignancies emphasize the context-dependent nature of AhR biology. Findings in SH-SY5Y may not generalize to all neuroblastoma lines or in vivo settings; future work should test additional models and dose ranges. Future studies are warranted to investigate the activity of CYP1A1, assess the expression of downstream AhR target genes, and evaluate mitochondrial function in response to each ligand, thereby providing a deeper understanding of the underlying mechanisms.

4. Conclusion

Taken together, our findings show that AhR ligands exert different and occasionally paradoxical effects in SH-SY5Y cells. These results highlight the dual and context-dependent roles of AhR in cancer biology, suggesting that selective targeting of AhR signaling may hold therapeutic potential in neuroblastoma.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

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Authors Contributions

Conceptualization: AS, MO; Methodology: HPP, AM, EKA, MAF, MJ; Writing original draft: AS, MO, HPP, AM, EKA, MAF, MJ. All authors approved the final manuscript.

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There was no use of artificial intelligence in the making of this article.

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