Effects of targeting highly expressed in cancer protein 1 (Hec1) inhibitor INH1 in breast cancer cell lines \textit{In Vitro}

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**ABSTRACT**

In the present study, the \textit{in vitro} antiproliferative effect of targeting highly expressed cancer protein 1 (Hec1) inhibitor INH1 was investigated in estrogen receptor-positive MCF-7 cell line originating from an in situ carcinoma and triple negative MDA-MB-231 cell line originating from metastatic carcinoma. Cell viability, xCELLigence RTCA DP instrument CI values, MI, BrdU proliferation assay, and AI analyses were employed for this purpose. According to the findings of the current study, INH1 altered cell proliferation by lowering cell viability, CI, MI values, and BrdU proliferation while raising AI values in both cell lines. Between the experimental and control groups, there were noticeable changes (p<0.05). These findings imply that INH1's mode of action is not dependent on the presence of estrogen receptors, making it a potentially effective therapy for breast cancer.

**Introduction**

Despite advances in the diagnosis and treatments of breast cancer, 20% of newly diagnosed patients die each year (1). To provide the most individualized, secure, and effective therapy, treatment options are dependent on the grade, stage, and BC molecular subtype (2). Approximately 15% of diagnosed breast cancers worldwide are insensitive to hormone-based treatments. This type of cancer with a poor prognosis is negative in terms of estrogen receptor (ER), progesterone receptor (PR) and Her 2 gene expression and has high metastatic potential (3). In contrast, Her2 gene expression is not present in Luminal A breast cancer, despite the fact that it expresses the ER and PR. It has low clinical staging and a low cell proliferation index. Luminal A breast cancer with a good prognosis has similar features to normal breast cells and is the most common among breast cancers (4).

Through a variety of signal effectors, NEK2 has significant oncogenic effects in malignancies. Thus, targeting NEK2 while coordinating chemotherapy, radiation, and immunotherapy is a viable method for treating cancer. It provides a fresh way to use monotherapy to achieve group effectiveness. Adrenocortical carcinoma, liver HCC, and lung adenocarcinoma are just a few examples of the many NEK2 dominant cancer types that potentially benefit from NEK2-targeted therapy approaches (5). Hec1 is a subunit of the kinetochore-associated Ndc80 complex and is highly expressed in cancer protein 1. This complex structure is involved in the proper separation and alignment of sister chromatids by attaching microtubules to the kinetochore in the mitotic division (6, 7). Hec1 is upregulated throughout the cell cycle and recruited into the kinetochore (8). Its activity is regulated by Aurora B and Nek2 (9). The ability of Hec1 to operate during mitosis and the survival of cells depend on Nek2 and Aurora B regulation (6, 9, 10). Hec1 dysfunction results in aberrant mitotic processes that cause apoptotic cell death (10).

Hec1 is more expressed in rapidly dividing cells than in slow dividing cells. Its expression increases as a result of the transformation of cells. Hec1 is overexpressed in human cancers, including lung, breast, cervical, colorectal, liver, brain, and gastric cancers (10, 11, 12).

In this experimental study, it was aimed to evaluate the effects of the Hec1 inhibitor INH1 on breast cancer cell lines with different molecular subtypes at the cellular level.

**Materials and Methods**

**Cell culture**

Our research laboratory purchased triple-negative breast cancer model MDA-MB-231 and Luminal A breast cancer model MCF-7 cell lines from the European Cell Culture Collection (CCL). DMEM containing 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) was used to grow the cells.

**Cell Viability**

MCF-7 and MDA-MB-231 cell lines were seeded into 96-well plates at 10^4 cells per well. After an overnight incubation, 20 μM, 40 μM and 80 μM concentration of INH1 for MCF-7 and 40 μM, 50 μM and 60 μM concentration of INH1 for MDA-MB-231 were applied to the cells for 24 hours. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide) assay was performed at 24 h. The absorbance values of all experimental groups were measured...
with a spectrophotometer at 490 nm.

**Cell Index (CI)**

100 µl of DMEM was added to each well of 16-well e-plates containing microelectrodes and background reading was performed. Then, cell count was performed and 1000 cells for MCF-7 and 5000 cells for MDA-MB-231 were seeded in each well. Each well’s ultimate capacity was 200 L. For up to 24 hours without treatment and 72 hours after treatment with various doses of INH1, 16 well E-Plates were incubated under appropriate cell culture conditions and monitored on the RTCA system at 15-minute time intervals. Changes in cell proliferation have been noticed in the E-Plates incubator (owned by Roche Diagnostics GmbH, Penzberg, Germany).

**Mitotic Index (MI)**

Cells from both cell lines were plated in 24-well plates with 3x10^4 cells each to determine the percentage of mitotic cells. Cells underwent a 24-hour incubation period after seeding. At the conclusion of the experimental periods, cells treated with the optimal INH1 concentration were fixed using Carnoy’s fixative. After that, the Giemsa staining and Feulgen procedure were used. For each experimental group, 3000 cells were roughly counted using a light microscope to analyze MI.

**BrdU Proliferation Assay**

Following injection of the IC_{50} dose of INH1, the DNA synthesis rate of MCF-7 and MDA-MB-231 cells was assessed using the compound 5-bromo-2’-deoxyuridine (BrdU). Following the manufacturer's instructions, BrdU was produced, and then it was found using a spectrophotometric technique.

**Apoptotic Index (AI)**

To identify the presence of apoptotic cells, DAPI staining was used after INH1 administration. The cells were fixed after the experimental period and then treated with DAPI, a fluorescent dye, for 20 min. A fluorescent microscope was used to count apoptotic cells after staining.

**Statistical Analysis**

The data of the experimental groups were compared with a one-way ANOVA test. Statistical Analysis, Graphpad Prism version (Graphpad Software, San Diego, California, USA) has been done. A p <0.05 significance level was accepted in the tests.

**Results**

**Cell Viability**

The absorbance values of the MCF-7 cell line for INH1 were 266,13 x10^{-3}, 197,14 x10^{-3}, 140,98 x10^{-3} and 98,38 x10^{-3} respectively for control, 20 µM, 40 µM and 80 µM for 24 h (Figure 1). These values were 318,24 x10^{-3}, 157,15 x10^{-3}, 108,76 x10^{-3} and 95,54 x10^{-3} respectively for control, 40 µM, 50 µM and 60 µM for MDA-MB-231 cells for 24 h (Figure 2).

When these absorbance values were examined, compared to the 100% accepted control group for MCF-7 cells, it was observed that 20 µM INH1 concentration decreased cell viability to 74.07%, 40 µM INH1 concentration decreased cell viability to 52.97% and 80 µM INH1 concentration decreased cell viability to 36.96% (Figure 3). For MDA-MB-231 cells, it was observed that 40 µM INH1 concentration decreased cell viability to 49.38%, 50 µM INH1 concentration decreased cell viability to 34.17% and 60 µM INH1 concentration decreased cell viability to 30.02% (Figure 4). According to the data obtained, it was seen that 40 µM INH1 concentration for both cell lines was the approximate IC_{50} concentration.

**Cell Index (CI)**

The standard curves were used to compare the cell survival with a spectrophotometer at 490 nm.

![Figure 1. Absorbance values of mitochondrial dehydrogenase activity (450-690 nm) of MCF-7 cells treated with 20 µM, 40 µM, and 80 µM concentrations of INH1 for 24 h (p<0.05).](image1)

![Figure 2. Absorbance values of mitochondrial dehydrogenase activity (450-690 nm) of MDA-MB-231 cells treated with 40 µM, 50 µM, and 60 µM concentrations of INH1 for 24 h (p<0.05).](image2)

![Figure 3. Percent viability values of MCF-7 cells treated with 20 µM, 40 µM, and 80 µM INH1 for 24 h (p<0.05).](image3)
index values that were obtained by administering INH1 to MCF-7 cells at doses of 20, 40, and 80 M and MDA-MB-231 cells at 40, 50, and 60 M. INH1 demonstrated an antiproliferative impact on the MCF-7 and MDA-MB-231 cell lines, according to the results. However, various doses triggered various cell death pathways. Figures 5 and 6 show that while for the MCF-7 cell line, all concentrations had a cytostatic effect, for MDA-MB-231 cells all concentrations had an anti-mitotic effect. Additionally, using the xCelligence DP device's system, the IC$_{50}$ values of MCF-7 and MDA-MB-231 cells were found to be 43 M and 40 M precisely, respectively, after applying various doses of INH1 to these cells (Figures 7 and 8).

**Mitotic Index (MI)**

To assess changes in the proportion of cells that are in the mitotic phase, the values obtained by applying the optimum concentration of INH1 respectively 43 μM and 40 μM to hormone-sensitive MCF-7 and hormone-insensitive MDA-MB-231 cells are shown in Tables 1 and 2.

**BrdU Proliferation Assay**

To assess changes in the proportion of cells that are in the synthesis phase, the values obtained by applying the optimum concentration of INH1 respectively 43 μM and 40 μM to hormone-sensitive MCF-7 and hormone-insensitive MDA-MB-231 cells are shown in Tables 3 and 4.

**Apoptotic Index (AI)**

In order to determine the apoptotic effect of INH1 on hormone-sensitive MCF-7 and hormone-insensitive MDA-MB-231 cells, respectively 43 μM and 40 μM INH1 were applied for 0-72 h. The values of the increase in apoptotic cells following administration of these concentrations are shown in Tables 5 and 6.

**Discussion**

With current standard treatment approaches, it is difficult to control especially advanced breast cancer cases. Therefore, the need to develop different treatment strate-

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<th>Time (h)</th>
<th>Control</th>
<th>43 μM</th>
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<tbody>
<tr>
<td>24 h</td>
<td>4,2 ± 0,03</td>
<td>2,43 ± 0,02</td>
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<tr>
<td>48 h</td>
<td>4,8 ± 0,02</td>
<td>2,04 ± 0,01</td>
</tr>
<tr>
<td>72 h</td>
<td>5,3 ± 0,03</td>
<td>1,27 ± 0,01</td>
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**Table 1.** Mitotic index (%) values of MCF-7 cells treated with 43 μM of INH1 for 24, 48 and 72 h (p<0.05).

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<thead>
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<th>Time (h)</th>
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<tr>
<td>24 h</td>
<td>3,19 ± 0,02</td>
<td>2,08 ± 0,03</td>
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<tr>
<td>48 h</td>
<td>3,68 ± 0,02</td>
<td>1,73 ± 0,01</td>
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<tr>
<td>72 h</td>
<td>4,42 ± 0,03</td>
<td>1,04 ± 0,01</td>
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**Table 2.** Mitotic index (%) values of MDA-MB-231 cells treated with 40 μM of INH1 for 24, 48 and 72 h (p<0.05).

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<th>Time (h)</th>
<th>Control</th>
<th>43 μM</th>
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<tbody>
<tr>
<td>24 h</td>
<td>100</td>
<td>53</td>
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<tr>
<td>48 h</td>
<td>100</td>
<td>43</td>
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<tr>
<td>72 h</td>
<td>100</td>
<td>27</td>
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**Table 3.** % BrdU values of MCF-7 cells treated with 43 μM of INH1 for 24, 48 and 72 h (p<0.05).

<table>
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<tr>
<th>Time (h)</th>
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<tr>
<td>24 h</td>
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<td>51</td>
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<tr>
<td>48 h</td>
<td>100</td>
<td>41</td>
</tr>
<tr>
<td>72 h</td>
<td>100</td>
<td>32</td>
</tr>
</tbody>
</table>

**Table 4.** % BrdU values of MDA-MB-231 cells treated with 40 μM of INH1 for 24, 48 and 72 h (p<0.05).
Effects (10).

Hec1 is overexpressed in several cancer subtypes, and this overexpression causes the mitotic checkpoint to become hyperactive (10, 16, 17). Numerous cancer types have higher Hec1 expression, which is linked with a poor prognosis (18-20). Hec1 mRNA is highly expressed in various malignancies. Additionally, increased Hec1 protein levels have been found in clinical tumor samples and have been linked to tumor grade and the prognosis of primary breast cancer (10).

Because medications that target this protein induce widespread chromosomal aneuploidy and cell death in cancer cells, Hec1 is a viable molecular target for the development of novel therapeutic treatments (21).

A tiny molecule called INH1 binds to HEC1 directly and breaks down the HEC1/NEK2 connection. This binding causes NEK2 to degrade, which ultimately results in cell death. In breast cancer cell lines, INH1 has been demonstrated to successfully limit cell growth and has been linked to tumor grade and the prognosis of primary breast cancer (10).

Another Hec1 inhibitor, TAI-1, has been proven to induce apoptotic cell death and significantly slow tumor development in cancer xenograft models in research examining its efficacy. An in vitro and in vivo study examining the effectiveness of TAI-95, a Hec1 inhibitor, in treating primary liver cancer found that it outperformed sorafenib and had a substantial antiproliferative effect on several primary liver cancer cell lines. Nine out of the 11 breast cancer cell lines tested in a study looking at the effectiveness of TAI-95, a Hec1 inhibitor, in treating breast cancer cell lines generated from luminal A and triple-negative breast cancer, respectively, have antiproliferative effects when treated with the Hec1 inhibitor INH1.

This study demonstrated that two different breast cancer cell lines, MCF-7 and MDA-MB-231, are nanomolar sensitive to this inhibitor (24-27). Of the Hec1 inhibitor TAI-95 on breast cancer were shown to successfully limit cell growth (10, 22). The INH1 suppressed cell proliferation under in vitro settings between 10 and 21 μM. Additionally, INH1 treatment inhibited tumor development in a nude mice model carrying xenografts generated from the human breast cancer line MDA-MB-468 without causing any obvious negative effects (10).

Table 5. Apoptotic index (%) values of MCF-7 cells treated with 43 μM of INH1 for 24, 48 and 72 h.

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<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tbody>
<tr>
<td>Control</td>
<td>2,14 ± 0,02</td>
<td>3,15 ± 0,03</td>
<td>4,13 ± 0,03</td>
</tr>
<tr>
<td>43 μM</td>
<td>8,17 ± 0,04</td>
<td>15,16 ± 0,04</td>
<td>22,43 ± 0,06</td>
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</table>

Table 6. Apoptotic index (%) values of MDA-MB-231 cells treated with 40 μM of INH1 for 24, 48 and 72 h.

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<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3,16 ± 0,02</td>
<td>3,89 ± 0,03</td>
<td>4,74 ± 0,03</td>
</tr>
<tr>
<td>40 μM</td>
<td>10,12 ± 0,05</td>
<td>17,46 ± 0,05</td>
<td>26,18 ± 0,07</td>
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In this study, the fact that INH1 had antiproliferative effects on ER (+) luminal A breast cancer cell line MCF-7 and ER (-) TNBC cell line MDA-MB-231 supports previous studies in the literature and indicates that the Hec1 inhibitor INH1’s mode of action is unrelated to the estrogen receptor’s presence.

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Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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