In vitro anticancer activity of N-benzyl 1,10-phenanthroline derivatives on human cancer cell lines and their selectivity

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ABSTRACT This research was conducted to evaluate the anticancer activity of new compounds of benzyl-1,10-phenanthroline derivatives and their selectivity. In vitro anticancer activity of 11 benzyl-1,10-phenanthroline derivatives were conducted on three human cancer cell lines, cervical cancer (HeLa), myeloma (NS-1), and breast cancer (MCF-7) using MTT-based cytotoxicity assay. The cytotoxicity of each compound was assessed to normal Vero cell line by the same method. The in vitro anticancer activity and cytotoxicity was expressed by the concentration inhibiting 50% of the cell growth (IC50), and the selectivity index (SI) was determined by calculating ratio of the IC50 on Vero cell line and the human cancer cell lines. The results showed that among the 11 compounds tested, the (1)-N-(4-butoxybenzyl)-1,10-phenanthrolinium bromide possessed a potential in vitro anticancer activity with an IC50 27.60 ± 2.76 µM on HeLa, 6.42 ± 5.53 µM on NS-1 and 9.44 ± 2.17 µM on MCF-7 cell lines. Its SI were 377.65 ± 39.97 on HeLa, 6158.72 ± 5306.34 on NS-1 and 1140.11 ± 261.85 on MCF-7 cell lines. This study demonstrated that (1)-N-(4-butoxybenzyl)-1,10-phenanthrolinium bromide possessed a potential in vitro anticancer activity on cancer cell lines with high selectivity.

KEYWORDS cytotoxicity; human cancer cell lines; in vitro anticancer activity; selectivity index

1. Introduction

Many studies have been conducted to find new compounds to be developed as anticancer drugs. The increased dependence of tumor cells on iron has led to the suggestion that depleting iron may be a strategy to limit tumor growth (Huang 2003). Potent Fe chelator, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone demonstrated selectivity against cancer cells compared with normal cells in vitro (Chaston et al. 2003). Metal chelators and metal ions have been shown to induce apoptosis through reactive oxygen species (ROS) generation (Duan et al. 1999), and act as effective antiproliferative agents (Darnell and Richardon 1999; Gao and Richardon 2001).

The iron chelators have become a target for the development of anticancer drugs (Richardson 2002). Iron is one of the essential elements for the existence of cancer cells. Iron ions play an important role in several cell growth processes, mainly the DNA synthesis. Without iron a cell cannot finish its cycle and proceed from the G1 phase to S (Nyholm et al. 1993; Huang 2003).

The anticancer activity of an iron chelator which has been studied previously is desferrioxamine (DFO). Research has proven that DFO can obstruct lymphoma growth in animals, both in vitro (Kemp et al. 1990) and in vivo (Kemp et al. 1995). Research has also shown that DFO strongly obstructs neuroblastoma growth (Donfrancesco et al. 1990). However, the use of DFO is still very limited because of its limited membrane permeability, poor absorption from the colon, and short half life in plasma. It also needs to be used in a long term subcutaneous infusion (for 12–24 hours per day and 5–6 times per week) and is therefore costly (Richardson 2002). The DFO also has genotoxic effects (Kim et al. 2007). As a result, recently there have been numerous attempts to identify another iron chelator which can be developed specifically for cancer treatment. One group of potential agents is 1,10-phenanthrolines.
ring system is well known for its metalloproteinase inhibition by chelating divalent metal ions. As a metal chelating compound, 1,10-phenanthroline has been used as antimicrobial agent against bacterial species such as \textit{Prevotella ruminicola}, \textit{Fibrobacter succinogenes}, \textit{Lachnospira multipara}, and \textit{Megasphaera elsdenii wallace1996}. The antimarial activity of 1,10 phenanthroline was reported by Yapi et al. (2000), and increased after blocking of the potential chelating site by $N$-alkylation. Some compounds of $N$-alkyl and $N$-benzyl 1,10-phenanthroline derivatives have shown antiplasmodial activity against FCR-3 and D10 \textit{Plasmodium falciparum} (Sholikhah et al. 2006), and antiplasmodial activity in mouse malaria models (Wi-jayanti et al. 2006).

The antitumor activity of 1,10-phenanthroline was reported by Sakurai et al. (1995). A derivative 1,10 phenanthroline, bis (4,7-dimethyl-1,10-phenanthroline) sulfatooxovanadium (IV) induced apoptosis in human cancer cells (Narla et al. 2000), exhibited antileukemic activity with matrix metalloproteinase inhibition (Narla et al. 2001b), significant antitumor activity and delayed tumor progression in CB.17, combined severe immunodeficient (SCID) mouse xenograft models of human glioblastoma and breast cancer (Narla et al. 2001a).

In the previous research, six compounds of $N$-benzylated 1,10-phenanthroline derivatives were synthesized and their cytotoxic activities were tested. The $N$-benzylated 1,10-phenanthroline expected that they do not have metal chelating activity. However, among the six tested compounds, the derivatives of benzyl-1,10-phenanthroline: (1)-$N$-benzyl-1,10-phenanthroline iodide and (1)-$N$-(4-benzyloxy-3-methoxybenzyl)-1,10-phenanthroline chloride have cytotoxic activities with $IC_{50}$ at the myeloma cell lines of 12.15 ± 1.31 and 2.39 ± 0.27 \textmu M, and on the HeLa cell lines of 8.57 ± 0.58 and 4.85 ± 0.31\textmu M (Sholikhah et al. 2007). It suspected another mechanism of their anticancer activity. In a subsequent research, several new compounds of benzyl-1,10-phenanthroline derivatives have been successfully synthesized. However, the anticancer activities of these synthesized compounds have not yet been studied. This research was conducted to evaluate the anticancer activities of new compounds of benzyl-1,10-phenanthroline derivatives upon several cancer cell lines and their selectivity.

2. Materials and methods
2.1. Compounds Tested
There are 11 new compounds of benzyl-1,10-phenanthroline derivatives have been synthesized (Figure 1). Figures 1a, 1b, and 1c are the same compound with different salt forms. Similarly, Figures 1d, 1e, and

![FIGURE 1](image-url)
1f are the same compound, and Figures 1g and 1h are the same compound, with different salt form. Doxorubicin HCl (Ferron Par Pharmaceuticals, Indonesia) was used as a positive control.

2.2. Cell lines
In vitro anticancer activity of the compounds was assessed against three human cancer cell lines: a cervical cancer (HeLa) cell line, myeloma (NS1) cell line, and breast cancer (MCF-7) cell line which were obtained from Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia. The human cancer cell lines were maintained in vitro in RPMI-1640 medium (Sigma-Aldrich Inc., USA), supplemented with 7.68 mM of HEPES (Sigma Chemical Co., USA), containing 10% fetal bovine serum (Gibco Invitrogen, USA), 2% penicillin-streptomycin (Gibco Invitrogen, USA), and 0.5% fungizone (Gibco Invitrogen, USA) in tissue culture flask.

To determine the Selectivity Index (SI), the compounds were assessed against Vero cell line obtained from Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Indonesia. The Vero cell line was cultured in M199 medium (Gibco, Auckland) containing 10% fetal bovine serum (Gibco Invitrogen, USA), 0.5% fungizone (Gibco Invitrogen, USA) in phosphate buffer saline.

2.3. In vitro anticancer activity and cytotoxicity assessment
The cell was cultured in 96-well plates at 2 × 10^4 cells/well in 100 µL medium and were incubated in 5% CO₂ incubator at 37°C for 24 h. A solution (100 µL) of a compound or doxorubicin was added at six concentrations and were incubated for 24 h. The first concentration of the compound 1 mg/mL was diluted with cell medium to obtain six concentrations of the compound. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based cytotoxicity assay (Freshney 2000) was used to evaluate the in vitro anticancer activity and cytotoxicity of the compounds. After the incubation period was finished, culture medium was taken and the cells were resuspended in new medium and 10 µL solution of 5 mg/mL of MTT (Sigma Chemical Co., USA) was added, then incubated for 4 h. The formation of formazan from MTT was interrupted by adding a 100 µL of 0.04 M HCl-isopropanol. Optical density (OD) of formazan was read by ELISA reader at λmax 550 nm. The value of OD is directly proportional to the number of live cells. The cell culture without tested compounds was used as a control, considered to have 100% growth. In vitro anticancer activity and cytotoxicity was expressed as IC₅₀ which was determined by probit analysis based on the relation between the concentration of the tested compound and the percentage of cell growth inhibition. The mean and standard deviation of the IC₅₀ was calculated from three replication.

2.4. Selectivity index assessment
Selectivity index of each compound was determined by calculating the ratio of IC₅₀ values obtained with Vero cell line and a cancer cell line, multiplied by 100.

3. Results and discussion
3.1. In vitro anticancer activity assessment
Table 1 showed the in vitro anticancer activity of 11 compounds of the 1,10-phenanthroline derivatives and doxorubicin HCl (Ferron Par Pharmaceuticals, Indonesia) were obtained after treatment with 0.125% trypsin (Gibco, Auckland) in phosphate buffer saline.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (means ± SEM, in µM)</th>
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<tbody>
<tr>
<td></td>
<td>HeLa cell line</td>
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<tr>
<td>(1)-N-benzyl-6-nitro-1,10-phenanthroline chloride (Figure 1a)</td>
<td>60.86 ± 23.72</td>
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<tr>
<td>(1)-N-benzyl-6-nitro-1,10-phenanthroline bromide (Figure 1b)</td>
<td>216.68 ± 59.99</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-nitro-1,10-phenanthroline iodide (Figure 1c)</td>
<td>232.39 ± 154.88</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-bromo-1,10-phenanthroline chloride (Figure 1d)</td>
<td>116.37 ± 12.70</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-bromo-1,10-phenanthroline bromide (Figure 1e)</td>
<td>71.83 ± 31.49</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-bromo-1,10-phenanthroline iodide (Figure 1f)</td>
<td>125.57 ± 50.01</td>
</tr>
<tr>
<td>(1)-N-(4-methoxybenzyl)-1,10-phenanthroline bromide (Figure 1g)</td>
<td>148.87 ± 92.68</td>
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<tr>
<td>(1)-N-(4-ethoxybenzyl)-1,10-phenanthroline chloride (Figure 1h)</td>
<td>60.07 ± 10.75</td>
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<tr>
<td>(1)-N-(4-ethoxybenzyl)-1,10-phenanthroline bromide (Figure 1i)</td>
<td>117.36 ± 50.13</td>
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<tr>
<td>(1)-N-(4-butoxybenzyl)-1,10-phenanthroline bromide (Figure 1j)</td>
<td>27.60 ± 2.76</td>
</tr>
<tr>
<td>(1)-N-(4-benzylxy-3-methoxybenzyl)-1,10-phenanthroline chloride (Figure 1k)</td>
<td>36.28 ± 7.92</td>
</tr>
<tr>
<td>Doxorubicin HCl (Figure 1l)</td>
<td>22.39 ± 5.95</td>
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</tbody>
</table>
TABLE 2 IC\textsubscript{50} of N-benzyl 1,10-phenanthroline derivatives on Vero Cell Line and its selectivity index (SI).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (means ± SEM, in µM)</th>
<th>Selectivity Index (SI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa cell line</td>
<td>Myeloma (NS1) cell line</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-nitro-1,10-phenanthrolinium chloride (Figure 1a)</td>
<td>393.84 ± 104.32</td>
<td>890.40 ± 336.78</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-nitro-1,10-phenanthrolinium bromide (Figure 1b)</td>
<td>452.74 ± 167.19</td>
<td>226.28 ± 62.65</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-nitro-1,10-phenanthrolinium iodide (Figure 1c)</td>
<td>220.78 ± 26.25</td>
<td>170.88 ± 113.88</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-bromo-1,10-phenanthrolinium chloride (Figure 1d)</td>
<td>177.22 ± 96.78</td>
<td>154.12 ± 16.82</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-bromo-1,10-phenanthrolinium bromide (Figure 1e)</td>
<td>252.28 ± 30.77</td>
<td>434.76 ± 190.61</td>
</tr>
<tr>
<td>(1)-N-benzyl-6-bromo-1,10-phenanthrolinium iodide (Figure 1f)</td>
<td>289.19 ± 60.73</td>
<td>302.21 ± 120.37</td>
</tr>
<tr>
<td>(1)-N-(4-methoxybenzyl)-1,10-phenanthrolinium bromide (Figure 1g)</td>
<td>188.67 ± 78.12</td>
<td>206.92 ± 128.82</td>
</tr>
<tr>
<td>(1)-N-(4-ethoxybenzyl)-1,10-phenanthrolinium chloride (Figure 1h)</td>
<td>103.80 ± 33.21</td>
<td>187.27 ± 40.53</td>
</tr>
<tr>
<td>(1)-N-(4-ethoxybenzyl)-1,10-phenanthrolinium bromide (Figure 1i)</td>
<td>136.76 ± 40.78</td>
<td>142.53 ± 60.88</td>
</tr>
<tr>
<td>(1)-N-(4-butoxybenzyl)-1,10-phenanthrolinium bromide (Figure 1j)</td>
<td>102.03 ± 16.46</td>
<td>377.65 ± 39.97</td>
</tr>
<tr>
<td>(1)-N-(4-benzylxylo-3-methoxybenzyl)-1,10-phenanthrolinium chloride (Figure 1k)</td>
<td>15.07 ± 15.05</td>
<td>43.61 ± 9.52</td>
</tr>
<tr>
<td>Doxorubicine (Figure 1l)</td>
<td>257.14 ± 82.40</td>
<td>1235.67 ± 328.50</td>
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</tbody>
</table>

* SI = IC\textsubscript{50} Vero cell line/IC\textsubscript{50} Cancer cell line x 100

bicine. The in vitro anticancer activity of all the 1,10-phenanthroline derivatives and doxorubicine is greater in the myeloma cells and MCF-7 cells than in the HeLa cell line. Figures 1a, 1b, and 1c are the same compound; however, they have different anion. The difference in anion of Cl\textsuperscript{-}, Br\textsuperscript{-}, and I\textsuperscript{-}, the difference in ease to bind with receptor in cell lines, so that they have the difference anticancer activity. According to Burger and Fiebig (2014), a compound tested is active in anticancer screening using cell line when the IC\textsubscript{50} lower than 30 µM. Among those three compounds, the compound in Figure 1a showed the highest anticancer activity on NS-1 and MCF-7 cell lines, and the compound Figure 1b showed no anticancer activity. The compound in Figure 1c showed a high activity on NS-1 and MCF-7 cell lines, but no activity on HeLa cell line. The difference cell line may cause the difference receptor for the compounds tested. So that it produces the difference response to the compounds. Figures 1d, 1e, and 1f are the same compound, with different salt form. Similarly with the compounds in Figures 1a, 1b, and 1c, the difference in anion of Cl\textsuperscript{-}, Br\textsuperscript{-}, and I\textsuperscript{-} showed the difference in anticancer activity. Among the three compounds, the compound in Figure 1e showed highest anticancer activity on MCF-7 cell line. The compound in Figure 1g, which has difference structure with the other compounds, showed anticancer activity on NS-1 cell line. The compounds in Figures 1h and Figure 1i have the same main structure, but different salt form, and showed different activity. The compound in Figure 1h active on NS-1 and MCF-7 cell line, however the compound in Figure 1i is active on NS-1 cell line only.

Among the 11 compounds, (1)-N-(4-butoxybenzyl)-1,10-phenanthrolinium bromide (Figure 1j) and (1)-N-(4-benzylxylo-3-methoxybenzyl)-1,10-phenanthrolinium chloride (Figure 1k) showed the highest anticancer activity on NS-1 and MCF-7 cell lines. According to their structure, these two compounds have high lipophilicity which may cause easier to penetrate through membranes.

3.2. In vitro cytotoxicity assessment and selectivity index determination

Table 2 presents the cytotoxicity of all 11 compounds and doxorubicine in normal Vero cell lines, and the Selectivity Index (SI) of each compound. According to Popiołkiewicz et al. (2005), a SI value of 100 or less would suggest that concentration of compound for achieving therapeutic effect is similar or lower than the concentration causing toxic effects. Obviously, the most desirable substances would have SI values greater than 100.

Among the 11 compounds tested, only compound in Figure 1k which has an SI smaller than 100, on HeLa cell line. The other 10 compounds have higher SI than 100,
but smaller than SI of doxorubicine as positive control. The (1)-N-(4-butoxybenzyl)-1,10-phenantroline bromide (Figure 1j) which has the highest anticancer activity showed has an SI greater than 100.

Phenanthroline is a tricycle aromatic hydrocarbon, with the formula C_{12}H_{8}N_{2}, and it is named 1,10-phenanthroline because two nitrogen atoms are found at C positions 1 and 10. It is also known as ortho-phenanthroline. The 1,10-phenanthroline is a typical metal chelator which can bind Fe(II) and prevent the hydroxyl radical formation which is mediated by Fe(II) through the Fenton reaction. This substance can also prevent DNA damage which is induced radical hydroxyl. Phenanthroline can activate the iron binding and activity of trans activation of DNA p53 in cancer cell of mouse in vitro. The activation of p53 is suspected to play a role in cell mortality because of apoptosis (Sun 1997).

The 1,10-phenanthroline has been proven to have some pharmacological activities. It has been shown to have fungicidal and fungistic actions, can breakdown the function of mitochondria, induce oxidative stress in yeast and mammalian cells, and have bacteriostatic and bactericidal effects (Coyle et al. 2004). Quaternary salts of 1,10-phenanthroline have studied and shown to have herbicide and carcinostatic activity (Dumitraşcu et al. 2004).

The study of a vanadium salt of 1,10-phenanthroline has shown that derivatives of 1,10-phenanthroline have cytotoxic effects. Narla et al. (2000) found that compounds with two ligand 1,10-phenanthroline have greater anticancer activity in low concentration. Bis(4,7-dimethyl-1,10-phenanthroline) sulfatooxovanadium (IV) (called METVAN) inhibit adhesion of leukemic cells on extracellular matrix, has anticancer activity in cancer cell lines of multiple myeloma, breast cancer, glioblastome, and testis cancer (Narla et al. 2001a, b).

In this research, the chelating capacity of 1,10-phenanthroline was blocked by N-10 benzylolation, so that the 11 compounds were expected that they do not have metal chelating activity. However, among the 11 compounds of benzyl-1,10-phenanthroline derivatives tested, only 1 compound, (1)-N-benzyl-6-nitro-1,10-phenanthroline bromide, has no anticancer activity. This result showed that the metalloprotease inhibition process is not correlated with their anticancer activity on three cancer lines used in this research. This finding suspected that there was another mechanism of their anticancer activity.

4. Conclusions

The (1)-N-(4-butoxybenzyl)-1,10-phenantroline bromide showed the best anticancer activity and has high selectivity on three cancer lines. However, its activity and selectivity are still lower than doxorubicine as positive control. Further investigation is required to find new compounds of benzyl-1,10-phenanthroline derivatives that have more potent activities and which are more selective than doxorubicine. Further studies should be conducted to evaluate in vivo anticancer activity in animal cancer models.

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Authors’ contributions

EN, J, and M designed the study. RH and J synthesized the compounds. EN carried out the laboratory work. EN, SW, and M analyzed the data. EN and M wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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