Synthesis, Anticancer Activity, Effect on Cell Cycle Profile, and Apoptosis-Inducing Ability of Novel Hexahydrocyclooctathieno[2,3-d]-pyrimidine Derivatives

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A novel series of hexahydrocyclooctathieno[2,3-d]pyrimidines was synthesized. Investigation of the anticancer activity of these derivatives revealed that compounds 2a and b showed broad-spectrum anticancer activity in nanomolar to micromolar concentrations. In particular, compound 2b showed a concentration required for 50% inhibition of cell growth (GI50) value of less than 1µM against 20 cancer cell lines. Compounds 2a and b induced G2/M- and S-phase cell cycle arrest in human colon adenocarcinoma (HCT116) and human breast adenocarcinoma (MCF7) cell lines with a concomitant increase in the pre-G cell population in a time-dependent manner. Furthermore, compound 2b increased the nuclear expression of the proapoptotic protein cleaved caspase-3, indicating that apoptosis has an important role, at least in part, in the cancer cell death induced by the new compounds.

Key words thieno[2,3-d]pyrimidine; synthesis; anticancer activity; cell cycle arrest; apoptosis; caspase-3

Apoptosis, or programmed cell death, is a major control mechanism by which cells die if DNA damage exceeds the capacity of repair mechanisms.1) As part of normal development, apoptosis plays an important role in controlling cell number and proliferation. Defects in apoptotic responses considered as a major contributor in different human diseases including cancer. Thus, development of novel drugs able to restore the normal apoptotic pathway, represents a new strategy in cancer treatment.2)

Recently, the thieno[2,3-d]pyrimidine core was incorporated in many potential anticancer agents.3–23) Wang et al. identified the thieno[2,3-d]pyrimidine 1 (Fig. 1) as potent anticancer lead.16) The preliminary structure–activity relationship study indicated that either 4-methoxyphenyl or 3,4,5-trimethoxyphenyl at C-2 position and the carbonyl group at C-4 position were essential for the anticancer activity. The cycloalkyl ring fused to thiophene was a major component for the antitumor activity that was affected by changing the size of the cycloalkyl ring. We reported in previous studies several thieno[2,3-d]pyrimidines with 8-membered cycloalkyl ring which showed potent anticancer activity.20–23) In an attempt to improve the potency of compound 1, we prepared new hexahydrocyclooctathieno[2,3-d]pyrimidines with 4-methoxyphenyl or 3,4,5-trimethoxyphenyl at C-2 position and different substituents at C-4 position. The in vitro anticancer activity of these thienopyrimidines was evaluated. For the most potent compounds, the mechanism of their antitumor activity, in terms of the effect on cell cycle distribution and induction of cellular apoptosis through the activation of pro-apoptotic protein, cleaved caspase-3 in the HCT116 and MCF7 cell lines was investigated.

Results and Discussion

Chemistry The synthesis of the target compounds is outlined in Chart 1. We obtained the starting material 1 according to the reported procedure24): Cyclocondensation of compound 1 with 4-methoxybenzaldehyde or 3,4,5-trimethoxybenzaldehyde in dry dimethylformamide afforded 2a and b. On refluxing 2a and b with phosphorus pentasulfide in xylene, the corresponding 4-thioxo derivatives 3a and b were obtained. Chlorination of 3a and b with phosphorus oxychloride afforded the corresponding 4-chloro derivatives 4a and b. The 4-hydrazinyl derivatives 5a and b were obtained through reacting compounds 4a and b with hydrazine hydrate in ethanol. Reacting compounds 4a and b with the appropriate primary amine in ethanol in the presence of of triethylamine produced the 4-substitutedaminothieno[2,3-d]pyrimidines 6a–f. Compound 1 was prepared according to the reported procedure20) to be used for comparison in biological evaluation.

Biological Evaluation

Anticancer Activity against HCT116 and MCF7 Cell Lines

The in vitro anticancer activity of all newly synthesized compounds was evaluated using HCT116 and MCF7 cell lines. Compound 1 and doxorubicin were used for comparison in this study. IC50 (concentration of the compound causing 50% inhibition of cell viability) was calculated.

Table 1 shows the IC50 of the synthesized compounds compared to doxorubicin and compound 1 and Fig. 2 presents the results graphically.

Anticancer Activity against a Panel of 56 Human Tumor Cell Lines

In this study, the National Cancer Institute (U.S.A.) selected compounds 2a and b for anticancer activity evaluation. They were evaluated against 56 cancer cell lines at 5 dose concentration levels. The relationship between percentage growth and log10 of sample concentration was plotted to obtain log10 GI50 (concentration required for 50% inhibition of cell growth). Table S1 shows the GI50 of compounds 2a

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and b. The results are presented graphically in Figs. S1 and S2. The results showed that both compounds 2a and b had potent broad-spectrum anticancer activity with GI<sub>50</sub> ranging between 0.107 and 8.17 µM. They exerted the highest selectivity against MDA-MB-435 melanoma cell line with GI<sub>50</sub> 0.107 and 0.115 µM. Compound 2b was observed to show marked broader anticancer activity with GI<sub>50</sub> less than 1 µM against 20 cancer cell lines. Moreover, it showed high potency and selectivity against melanoma and cancers of central nervous system (CNS), ovary as well as breast. It is worth mentioning

![Fig. 1. Potent Anti-proliferative Lead](chart1.png)

**Chart 1. The Synthetic Path and Reagents for the Preparation of the Target Compounds**
that compounds 2a and b exerted an improved IC$_{50}$ against HCT116 and MCF7 cell lines than compound I.

Cell Cycle Analysis and Detection of Apoptosis

The most active compounds, 2a and b, were selected to be further studied in terms of their effects on cell cycle progression and induction of apoptosis in the HCT116 and MCF7 cell lines, as a manifestation of their anti-proliferative action and their results were compared to compound I. HCT116 and MCF7 cells were incubated with IC$_{50}$ concentration of compounds 2a, b or I for 24 and 48 h and their effect on the normal cell cycle profile and induction of apoptosis were analyzed. Exposure of HCT116 and MCF7 cells to compounds 2a and b resulted in an interference with the normal cell cycle distribution on these cell lines. In HCT116, the 4-methoxyphenylthienopyrimidine 2a induced accumulation of cells in G2/M phase 6 folds and increased the percentage of cells in pre-G1 more than 2 folds with concomitant increase in S phase (2 folds). The trimethoxyphenyl analog 2b induced accumulation of cells in G2/M phase 15 folds. Similar results were observed following to trimethoxyphenylthienopyrimidine I treatment. The 4-methoxyphenylthienopyrimidine 2a induced 2-fold increase in the percentage of cells at pre-G1 and G2/M phases in MCF7 compared to control, while the trimethoxyphenyl analog 2b induced 2-fold increase in the percentage of cells at pre-G1 and 5-fold increase in percentage of cells in G2/M phases in MCF7 compared to control. Such increase was accompanied by a significant reduction in the percentage of cells at the G0 and G1 phase of the cell cycle. Similar results were observed following to compound I treatment. Accumulation of cells in pre-G1 phase, which was confirmed by the presence of a sub-G1 peak in the cell cycle profile analysis may have resulted from degradation or fragmentation of the genetic materials, indicating a possible role of apoptosis, while the accumulation of the cells in G2/M phase suggested induction of apoptosis.

Table 1. Concentrations Required for 50% Inhibition of Cell Viability (IC$_{50}$) for Compounds 2-5

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>IC$_{50}$ in µM</th>
<th>HCT116</th>
<th>MCF7</th>
</tr>
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<tbody>
<tr>
<td>2a</td>
<td>4-CH$_3$OC$_6$H$_4$</td>
<td>O</td>
<td>12.13 (1.39&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td>2b</td>
<td>3,4,5-(CH$_3$O)$_3$C$_6$H$_2$</td>
<td>O</td>
<td>11.07 (1.21&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td>3a</td>
<td>4-CH$_3$OC$_6$H$_4$</td>
<td>S</td>
<td>11.17</td>
</tr>
<tr>
<td>3b</td>
<td>3,4,5-(CH$_3$O)$_3$C$_6$H$_2$</td>
<td>S</td>
<td>46.63</td>
</tr>
<tr>
<td>4a</td>
<td>4-CH$_3$OC$_6$H$_4$</td>
<td>Cl</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4b</td>
<td>3,4,5-(CH$_3$O)$_3$C$_6$H$_2$</td>
<td>Cl</td>
<td>42.77</td>
</tr>
<tr>
<td>5a</td>
<td>4-CH$_3$OC$_6$H$_4$</td>
<td>NHNH$_2$</td>
<td>13.36</td>
</tr>
<tr>
<td>5b</td>
<td>3,4,5-(CH$_3$O)$_3$C$_6$H$_2$</td>
<td>NHNH$_2$</td>
<td>12.07</td>
</tr>
<tr>
<td>Compound I</td>
<td>2.67</td>
<td></td>
<td>3.00</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>8.71</td>
<td></td>
<td>9.06</td>
</tr>
</tbody>
</table>

<sup>a</sup> GI$_{50}$ (µM) by National Cancer Institute (U.S.A.).

Fig. 2. Graphical Representation of IC$_{50}$ Values of the Synthesized Compounds Compared to Doxorubicin and Compound I.
Fig. 3. Effect of Compounds 2a, b and I on DNA-Ploidy Flow Cytometric Analysis of HCT116 Cells

The cells were treated with IC_{50} (µM) of compounds 2a, b and I for 24 or 48 h, and the harvested cells were subjected to cell-cycle analysis using a FACS Calibur flow cytometer.

Fig. 4. Effect of Compounds 2a, b and I on DNA-Ploidy Flow Cytometric Analysis of MCF7 Cells

The cells were treated with IC_{50} (µM) of compounds 2a, b and I for 24 or 48 h, and the harvested cells were subjected to cell-cycle analysis using a FACS Calibur flow cytometer.
phase may have resulted from G2/M arrest (Figs. 3, 4). The 4-methoxy or 3,4,5-trimethoxy substitution pattern at the C-2 phenyl group of thienopyrimidine appear to have a considerable effect on the interference with the normal cell cycle distribution.

Effect on Cell Cycle Regulating Proteins

Because of subdiploid population of compound 2b-treated cells, we tested whether compound 2b can increase cleaved caspase-3 as a marker of apoptosis. Treatment of both MCF7 and HCT116 cancer cells with compound 2b increased the nuclear expression of cleaved caspase-3 (red fluorescence). The nuclear translocation of this protein confirms its activation (Figs. 5, 6), suggesting that activation of caspase-3 plays a pivotal role in compound 2b-induced cancer cell death, through induction of apoptosis. This effect was further confirmed by nuclear condensation and fragmentation as well as increase in cell membrane permeability and uptake of fluorescent stain 4',6'-diamidino-2-phenylindole (DAPI), leaving a stronger blue fluorescence, a characteristic feature of apoptosis.

Furthermore, as compound 2b treatment induced cell cycle arrest and increased G2/M population, we investigated whether the treatment stopped the cell growth in G2 or in M phase. Thus, the mitotic indicator-phosphohistone-H3 was used to differentiate between G2 and M population, following compound 2b treatment. Figures 5 and 6 showed that treatment of HCT116 cancer cells with compound 2b increased the nuclear expression of phosphohistone-H3 (green fluorescence) significantly compared to the non treated cells (Fig. 5) indicating that compound 2b stopped the HCT116 cell cycle at the M phase and halted its growth. Interestingly, treatment of MCF7 with compound 2b did not exert the same action on phosphohistone-H3 expression (Fig. 6). It is noteworthy that Compound 2b treatment had no effect on other cell cycle regulating proteins-cyclin B1 and cyclin D1 (data not shown).

Conclusion

In summary, we have successfully identified 2 hexahydrocyclooctathieno[2,3-d]pyrimidines 2a and b as broad-spectrum potent anticancer agents. Particularly, compound 2b exhibited broader anticancer activity. Both compounds induced G2/M and S phase cell cycle arrest in HCT116 and MCF7 cell lines with concomitant increase in the

Fig. 5. Fluorescence Photomicrographs Showing the Effect of Compound 2b on the Nuclear Expression of Cleaved Caspase-3, Phosphohistone-H3, and DAPI Staining in HCT116 Cells (A) Quantitation of Fluorescence Intensity (B)

AU=Arbitrary units. * Denotes significantly different from control at p<0.001.
pre-G cell population in time dependent manner. Furthermore, compound 2b increased the nuclear expression of the pro-apoptotic protein, cleaved caspase-3, indicating that apoptosis has an important role, at least partly, in the new compounds-induced cancer cell death.

**Experimental**

General chemistry methods, synthesis procedures, spectral data, biological assays, Tables S1 and S2, Figs. S1 and S2 are given in Supplementary information.

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**Conflict of Interest**  The authors declare no conflict of interest.

**Supplementary Materials**  The online version of this article contains supplementary materials.

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**References**